Characterization of differential Toll-like receptor function in human immune cells and association with susceptibility to recurrent HSV-1 reactivations and gastric cancer

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Summary

Toll-like Receptors (TLRs) are essential innate receptors which recognize conserved structures of pathogens, or danger signals released from damaged cells. Alterations of TLR responses might result in severe viral infections or a higher risk of cancer. Therefore, development of clinical assays to evaluate TLR functions could provide personalized information about susceptibility to these diseases. Since TLRs are differentially expressed on different subsets of human peripheral blood mononuclear cells (PBMCs), a multi-color flow cytometry-based assay was developed to detect TLR responses of individual cell types simultaneously. We observed that the magnitude of TLR responses largely varied between human subjects, but was highly reproducible over one month.

To evaluate the potential role of differences in natural killer (NK) cell TLR response we studied the association of NK cell TLR function and TLR single nucleotide polymorphisms (SNPs) with susceptibility to recurrent herpes labialis (HL) and gastric cancer. Using our assay, impaired TLR3 response of NK cells was found in people with recurrent HL. In addition, we have identified enhanced levels of homozygous TLR3 L412F SNP in people with recurrent HL, which results in lower surface expression and reduced NK cell response to poly(I:C).

TLR1 I602S, another common SNP, has been reported to decrease TNF-α responses of monocytes toward TLR2/1 agonist, Pam3CSK4 (Pam3Cys), stimulation. In our study, we found that TLR1 I602S homozygosity also contributes to impaired IFN-γ responses of NK cells and CD8+T cells. Although we did not observe an association of TLR2/1 deficiency with recurrent HL, association of TLR1 I602S with risk for primary as well as metastatic gastric cancer was found in a cohort of 326 patients.

To sum up, our results suggest that genetic polymorphisms of TLRs can impair TLR function of NK cells, which contribute to the increased susceptibility to HSV-1 diseases and gastric cancer.

Keywords: Toll-like receptor; Natural killer cells; Herpes simplex virus; Gastric cancer; Single nucleotide polymorphism

Zusammenfassung

Toll-like Rezeptoren (TLRs) sind essentielle angeborene Rezeptoren, die konservierte Strukturen von Krankheitserregern oder Gefahrsignale, die von beschädigten Zellen freigesetzt werden, erkennen können. Genetische Variationen in TLRs wie Einzel-Nukleotid-Polymorphismus (SNP) können die Funktion von TLRs beeinträchtigen und erste Studien zeigen, dass dies zu einer erhöhten Anfälligkeit gegenüber Virusinfektionen oder einem erhöhten Krebsrisiko führen kann. In dieser Studie haben wir einen Multicolor-Durchflußzytometrie-Test entwickelt, um die TLR-Funktionen in verschiedenen Subpopulationen unseparierter peripherer mononukleärer Blutzellen (PBMCs) simultan analysieren zu können. Wir konnten beobachten, dass das Ausmaß der TLR-Antworten zwischen den Probanden stark variierte, jedoch über einen Zeitraum von einem Monat gut reproduzierbar war.

Zunächst untersuchten wir TLR Reaktionen bei Patienten mit rezidivierenden Herpes labilalis (HL). Im Vergleich zu asymptomatischen Personen war eine HL- Anamnese mit einer signifikant verminderten TLR3-IFN-γ-Antwort nach Stimulation mit poly(I:C) in NK Zellen assoziiert. Weitere molekulare Untersuchungen zeigten eine mögliche Beteiligung von TLR3 L412F SNP, welcher die oberflächliche TLR3 Expression und die IFN-γ-Antworten in NK-Zellen reduzierte. Einige Studien zeigen, dassTLR1 I602S, ein weiterer sehr verbreiteter SNP, in der Lage ist die TNF-α-Antworten von Monozyten gegen den TLR2/1-Agonisten (Pam3Cys) zu verringern. In der hier vorliegenden Arbeit konnten wir zudem nachweisen, dass TLR1 I602S SNP auch die Funktion von NK-Zellen und CD8+ T-Zellen beeinträchtigt. Wir konnten keine Assoziation zwischen TLR2/1-Defizienz und reaktivierendem HL feststellen. Jedoch konnten wir an einer großen Kohorte von über 326 Patienten zeigen, dass der TLR1 SNP sowohl ein Risikofaktor für Magenkarzinomentstehung als auch für die Metastasierung ist.

Zusammenfassend weisen unsere Ergebnisse darauf hin, dass genetische Polymorphismen von TLRs die Funktion von NK-Zellen beeinträchtigen und zu einer erhöhten Anfälligkeit für HSV-1 Erkrankung und Magenkarzinom führen können.

Schlagwörter: Toll-like Rezeptoren; NK-Zellen; Herpes Simplex Virus; Magenkarzinom; Einzel-Nukleotid-Polymorphismus

Abbreviations

TLR: Toll-like receptor

HSV: Herpes simplex virus

HSE: Herpes simplex encephalitis

HL: Herpes labialis

PBMC: Peripheral blood mononuclear cell

NK: Natural killer cell

mDC: Myeloid dendritic cell

pDC: Plasmacytoid dendritic cell

APC: Antigen presenting cell

rhIL-12: Recombinant human interleukin-12

Pam3Cys: Pam3CSK4

BFA: Brefeldin-A

TNF-α: Tumor necrosis factor- α

IFN-γ: Interferon- γ

MFI: Mean florescence intensity

SNP: Single nucleotide polymorphism

APOE: Apolipoprotein E

NF-κB: Nuclear factor- κB

IKK: IκB kinase

1 Introduction

1.1 Overview of Toll-like receptors

Toll-like receptors (TLRs) are important innate immune receptors that recognize conserved features of microbes, or referred to as pathogen-associated molecular patterns (PAMPs). TLRs bind to PAMPs through solenoid ectodomains, which contain leucin-rich repeats; and exert inflammatory responses via pathways derived from the intracellular Toll-Interleukin-1 receptor (TIR) domain [1,2]. TLRs were named for their similarity to Toll, a receptor originally involved in embryonic development of the fruit fly *Drosophila melanogaster* discovered in 1985 by the German scientist Christiane Nüsslein-Volhard. In 1996, Jules A. Hoffmann and colleges further showed the importance of Toll in combating fungal infection in fruit flies [3]. Human TLRs were subsequently identified and shown to be involved in sensing specific structures of microbial origin and even endogenous danger signals (reviewed in [4]).

1.1.1 Toll-like receptors and viral infections

In humans, there are TLR1-10 in the TLR family. The activation of TLR during viral infection is essential for the induction of dendritic cell (DC) maturation, the production of interferons, and the initiation of the adaptive immune system [5].

1.1.1.1 TLR 1, 2, 4, 6

TLR 2 forms heterodimers with TLR1 or TLR6. Triacylated and diacylated lipoproteins, which are common gram-positive cell wall components, are ligands for TLR2/1 and TLR2/6. However, viral proteins of Herpes simplex virus -1, -2 (HSV-1, HSV-2), and Cytomegalovirus (CMV) have also been reported to be recognized by TLR2 [6,7]. Similarly, TLR4 is mainly activated by LPS, the component of the outer membrane of gram-negative bacteria, but it is also activated by the fusion protein of respiratory syncytial virus [8].

1.1.1.2 TLR 3, 7, 8, 9

TLR3, 7, 8 and 9 are specialized in the recognition of viral nucleic acids. These receptors reside in the endosome. TLR3 can be activated by double-stranded RNA (dsRNA) which is often produced during viral replication; TLR7 and 8 are stimulated by single-stranded RNA (ssRNA); and TLR9 is responsible for recognizing the nonmethylated CpG nucleotides in DNA viruses. For all TLR signaling except TLR3, the adaptor Myeloid differentiation primary response protein 88 (MyD88) is important for the activation of NF-kB pathways and thus the production of proinflammatory cytokines. TIR-domain-containing adapter-inducing interferon-β (TRIF), on the other hand, is involved in TLR3- and TLR4- mediated MyD88-independent pathways, and is essential for the induction of both NF-kB and type I interferon signaling [9].

1.1.2 Toll-like receptors and cancer

TLRs play several roles in carcinogenesis. For example, engagement of TLR2/1 complex significantly increases the anti-tumor activity of CD8T cells [10]. TLRs can also recognize endogenous signals released from damaged or dying tumor cells, thus breaking down tumor-induced immune tolerance [11]. Therefore, hypofunction of TLRs may increase risks for cancer. Furthermore, polymorphism in a promoter sequence of TLR2 and a TLR4 variant allele have been reported to be involved in Helicobacter pylori (H. pylori)-associated gastric cancer [12,13]. Polymorphism in the TLR10-TLR1-TLR6 gene cluster, on the other hand, is associated with an increased risk for prostate cancer [14].

1.2 Clinical problems

An overview of the clinical importance of recurrent HSV infections and roles of TLRs in HSV diseases is described below. Since TLR functions can also contribute to cancer risks, roles of TLR in susceptibility to gastric cancer are addressed at the end of this section.

1.2.1 Herpes simplex virus infections

1.2.1.1 Clinical aspects

Herpes simplex viruses are common human pathogens, which include herpes simplex virus -1 (HSV-1) and herpes simplex virus-2 (HSV-2). Primary infection or reactivation of HSV can cause a broad spectrum of diseases, such as gingivostomatitis in childhood, cold sores (herpes labialis), keratitis, genitalis, and even encephalitis. While Herpes labialis (HL) is the most prevalent manifestation of HSV-1 diseases, herpes simplex encephalitis (HSE) is the most common cause of sporadic viral encephalitis [15], which results in high morbidity and mortality. Moreover, genital co-infection of HSV and human immunodeficiency virus (HIV) can accelerate the disease progression to AIDS [16].

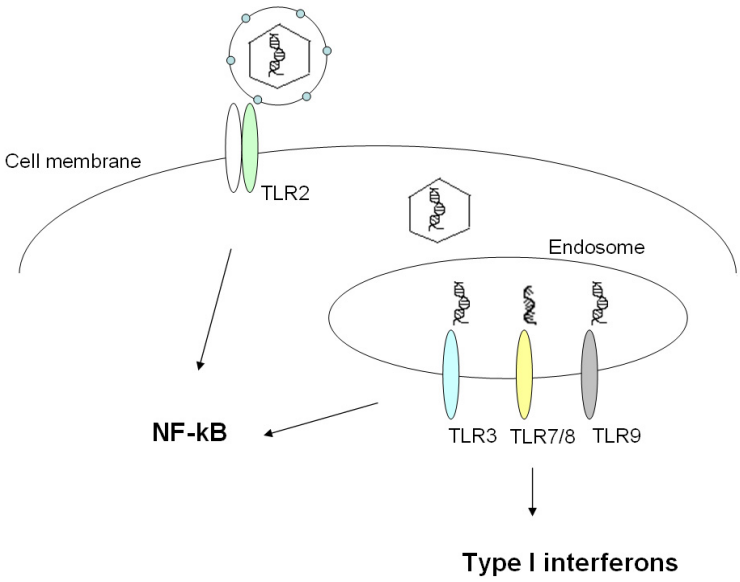
Once infected, the virus becomes latent in the neural ganglions, and reactivates from time to time. Although the guanosine analogue acyclovir which interferes with viral DNA replication can alleviate the disease, currently there is no biological method to prevent the primary infection of HSV, to prevent the establishment of latency, or to eradicate the latently-infected HSV. Vaccine developments for HSV are so far inconclusive (reviewed in [17,18]).

Toll-like receptor (TLR) agonists have emerged as an alternative for HSV treatment. For example, delivery of the TLR3 agonist poly(I:C) has been shown to increase the survival rate in a mouse model of HSE [19], and to protect human neural cells from HSV-1 infection in an in vitro study [20]. In addition, the TLR7 and TLR8 agonist, Resiquimod (R-848), has been applied as a cream in a small randomized controlled clinical study of genital HSV-2 infection, which decreased viral shedding rates [21]. However, large clinical trials are needed to determine the efficacy of treatment with TLR agonists.

1.2.1.2 Recognition of HSV-1 via TLRs

HSV belongs to Herpesviridae, which are a family of double-stranded DNA (dsDNA) viruses. HSV is in the subfamily of alpha-herpes viruses, and its linear DNA genome is encased within an icosahedral capsid, covered by tegument and envelope. TLRs are involved in the pathogenesis of HSV infections (Scheme. 1). It has been shown that the HSV protein can be recognized by TLR2 and the viral DNA can activate TLR9 signaling

[6]. The stimulation of TLR2 by HSV leads to NF-kB activation, and the stimulation of TLR9 leads to the production of type I interferons, especially in plasmacytoid dendritic cells (pDCs) (Reviewed by Finberg et al. [22]). Although HSV-1 is a dsDNA virus, it can produce single- and double-stranded RNA during viral replication. Thus, endocytosis of HSV-1-infected cells could make the recognition of viral nucleic acids via TLR3, 7 and 8 possible.



Scheme. 1: TLR recognition of HSV-1. It is proposed that some proteins on the surface of the virion can be recognized by TLR2. The double-stranded HSV-1 DNA viral genome can be recognized by TLR9 in the endosome, and RNAs produced during viral replication can be recognized by TLR3, 7 and 8. Double-stranded viral RNA can also be sensed by another innate receptor, RIG-I, which resides in the cytoplasm (not shown). The stimulation of the TLRs leads to the activation of NF-κB. Activation of TLR3, 7, 8 and 9 especially triggers the secretion of type I interferons in DCs. Type I interferons can further activate IFN-γ production in NK cells.

1.2.1.3 TLRs and susceptibility to recurrent HSV-1 diseases

HSV-1 infects 80-90% of all individuals by mid-life [23]. While most of the HSV-1 carriers are asymptomatic, approximately one third suffer from recurrent mucocutaneous infections - herpes labialis (HL). It is well known, that in HL-prone individuals potential immunosuppressive factors like stress, sun-exposure or infections can trigger the outbreak of HL. However, mechanisms underlying the difference of susceptibility to HL have been only partially elucidated. Recent findings provide evidence that genetic

factors may predispose to HL. Hobbs et al. has mapped a HL susceptibility locus on human chromosome 21 through linkage analysis [24]. Furthermore, it has been suggested that polymorphisms of apolipoprotein E-(APOE)-ε4, a molecule involved in HSV-1 viral entry, is associated with HL [25].

TLR3 mutation as well as deficiency in a TLR adaptor molecule UNC-93B resulting in impaired TLR3, TLR7, and TLR9 activation, were shown in otherwise clinically immunocompetent patients with severe HSV-1 reactivation, HSE [26,27]. It is possible that defects in TLR signaling also play a role in the susceptibility to recurrent HL.

1.2.2 TLRs and susceptibility to gastric cancer

Gastric cancer is one of the most common causes of cancer-related death in the world [28] and exhibits a clear inflammatory etiology [29,30]. Inadequate elimination of *H. pylori* leads to chronic gastritis and is the most recognized etiological risk factor for non-cardia gastric cancer [31,32]. However, *H. pylori* infection alone is not enough to cause gastric malignancy, since overall only 10% of infected people develop gastric or duodenal ulcers and less than 3% of the infected subjects develop gastric cancer [33]. Variations in host genetic factors involved in recognition and cytokine release upon *H. pylori* infection, such as TLRs, IL-1β and TNF-α have been reported to be associated with risks of gastric cancer (reviewed in [34]).

On the other hand, it has been shown recently that *H. pylori* induces innate immune response via NOD1 and the TLR-2/1 complex [35,36]. Moreover, dying tumor cells release high-mobility-group box 1 (HMGB1), which can stimulate the anti-tumor activity of immune cells via TLR2 [37]. Therefore, functional polymorphisms in TLR1 and TLR2 [38,39,40] might contribute to the susceptibility of gastric cancer.

1.3 Natural killer cell as an important player in viral infections and cancer

Natural killer cells (NK cells) play essential roles in the immunity to viral infections and malignant cells. NK cells are especially important in herpes virus infections, since NK-deficient patients usually have severe or frequent herpes diseases (as described below). Therefore, detecting potential defective TLR responses in NK cells derived from subjects with recurrent HSV-1 reactivations is one of the main focus in our study.

1.3.1 Introduction of natural killer cells

Natural killer cells (NK cells) are derived from lymphoid progenitors in the bone marrow and circulate in the peripheral blood. In the early phase of several intracellular infections, particularly with herpes viruses and Leishmania, NK cells are profiled by accessory cell-secreted cytokines, like type I interferons, IL-12, and TNF-α, to produce large amounts of IFN-γ. This is essential for controlling microbes before subsequent IFN-γ production of T cells. In addition, using a variety of invariant receptors, NK cells are able to recognize the changes of cell surface proteins induced by malignant transformation or viral infections, and kill the targets via the release of cytotoxic granules (summarized in [41]). There are two main subpopulations of NK cells: $CD56^{dim}$ NK cells consist of more than 90% of the NK cells, and are responsible for the cytotoxic activities; $CD56^{bright}$ NK cells are the minor subpopulation, and are mainly cytokine producers [42,43,44].

1.3.2 NK cells and herpes virus infections

NK cells play an important role in the control of chronic herpes virus infections (reviewed by Mossmann et al. [45]). For example, NK cells can directly recognize a protein of another herpes virus, mouse cytomegalovirus (MCMV) [46]. Defects in NK cell IFN-γ production or cytotoxicity have been reported to result in increased susceptibility to MCMV [47,48]. Furthermore, mice lacking NK cells are more susceptible to both HSV-1 and HSV-2 infections [49,50,51]. In human NK cell deficiency syndromes patients are particularly susceptible to recurrent or severe herpes virus diseases (including HSV, Epstein-Barr virus (EBV), Varicella zoster virus (VZV), and Cytomegalovirus (CMV)) [52,53]. In fact, NK cells are not only important in the early innate immune control of HSV, but are also reported to augment effective adaptive immune response against HSV [49].

1.3.3 Activation of NK cells via TLRs

NK cells recognize their targets by interplay of activating and inhibitory signals. For example, decreased MHC class I expression or increased amount of stress-induced NKG2D-ligands can lead to the activation of NK cells (reviewed in [54]). TLRs could serve as activating receptors of NK cells. Human NK cells have been reported to

express all known TLR mRNAs (TLR1-10), with higher expression levels of TLR2, 3, 5, and 6 [55,56]. Ligands for TLR2, 3, 4, 5, and 7 were shown to stimulate IFN-γ secretion of NK cells in the presence of a maintenance dose of IL-2 or IL-12 [56,57]. As described in the previous section, TLR2 and TLR9 have been shown to trigger the DC response to HSV-1 [6], and one of the HSV-1 genes is involved in controlling TLR3 responses [58]. Therefore, impairments of TLR responses in NK cells may result in increased susceptibility to recurrent or severe HSV-1 diseases. Indeed, Zhang et al.[26] recently demonstrated that NK cells isolated from two TLR3-deficient patients with HSE displayed impaired IFN-γ response to TLR3 agonist (poly(I:C)) stimulation, while their blood DCs responded normally in terms of IFN-α, -β, -γ secretion. However, TLR responses of NK cells in UNC-93B-deficient HSE patients have not been studied.

1.3.4 NK cells and tumor immune surveillance

NK cells and T cells can recognize tumor cells via cancer-induced molecules (NKG2D ligands) or tumor-specific antigens. Malignant cells might be eliminated by direct cytotoxic activities of these lymphocytes. In addition, IFN-γ produced by NK/T cells is an essential anti-tumor cytokine, as mice deficient in IFN-γ have been reported to develop spontaneous lymphoma and have increased incidence of lung adenocarcinoma [59]. Furthermore, NK cells also contribute to tumor immune surveillance by the induction of efficient T cell-mediated anti-tumor immune responses via the release of IFN-γ and priming of DCs [60]. In our study, a TLR single nucleotide polymorphism (SNP) was found to affect the IFN-γ production of NK cells and T cells. The association of the TLR SNP with risk of gastric cancer was examined in the last part of my project.

1.4 Evaluation of TLR function in different subsets of PBMCs

TLRs are differentially expressed on different subsets of human PBMCs. Most of the data obtained so far were done by examining the TLR mRNA level on resting isolated cell populations. We should be aware of the possibility that the expression of some TLRs might be up-regulated via the interaction between immune cell subsets in the *in vivo* physiology. In order to evaluate TLR responses of each immune cell type simultaneously, a multi-color flow cytometric assay was developed in our study to assess TLR function in unseparated cells *ex vivo*.

1.4.1 TLR expression on antigen-presenting cells

Studies of purified human monocytes, myeloid dendritic cells (mDCs), and plasmacytoid dendritic cells (pDCs) revealed differential expression of TLRs among each subset [55,61,62,63]. Freshly isolated human monocytes express TLR1, 2, 4, 5, 6 and 8, and mDCs express TLR1, 2, 3, 5, 6, 8, and 10 (reviewed in [61]). Although TLR4 expression on mDC is very low, it has been reported that the TLR4 agonist LPS induces IL-12 production by most mouse and human $CD11c^{high}$ DC subsets [63]. DCs are professional antigen-presenting cells (APCs), and in humans, mDC and pDC are the two main subtypes. Unlike mDCs, isolated human pDCs express only TLR1, 6, 7, 9, and 10 [55]. Both types of the DCs can respond to TLR stimulations by producing the inflammatory cytokine TNF-α, but pDC precursors, in particular, produce in addition a large amount of type I interferons in response to viral infections [64]. The two types of DCs do interact with each other. For example, type I interferons derived from pDCs are known to be instrumental in activating mDCs [65].

1.4.2 TLR expression on lymphocytes

TLR functions have been characterized in cells in the adaptive immune systems [66,67,68,69]. B cells express TLR1, 2, 6, 7, 9, 10, and possibly TLR4 and 8 [70]. On the other hand, B lymphocytes can be important APCs, and they respond to TLR2, 4, and 9 stimulations by producing IL-6, especially in conjunction with CD40 activation [71]. TLR 7 and 8 agonist R-848 has also been recognized as a potent B cell stimulator [68]. Similarly, T cells, another essential component of the adaptive immune system, express several TLRs [55]. MacLeod H and Wetzler LM have written in a perspective article [72] that the increasing data which demonstrate expression and activation of TLRs on T cells, provide evidence for a direct role for TLRs in the activation of an adaptive immune response. In fact, Imanishi et al. [73] have shown in mice that the stimulation of TLR2 on one type of effector T cells, Th1 cells, directly induced IFN-γ production, cell proliferation, and cell survival without T cell receptor (TCR) stimulation, and these effects were greatly enhanced by IL-2 or IL-12. Human NK cells also express a broad range of TLRs as described in the previous paragraphs.

1.4.3 Previous assays for evaluating TLR functions

Genetic defects and polymorphisms in TLR signal transduction have been reported to be related to severe viral and bacterial infections [27,74,75,76]. However, people with these diseases can show normal quantitative cellular parameters in the current clinical assays for innate and adaptive immunity [26,27,77]. Therefore, it is necessary to develop a functional assay to detect potential impairments of TLR signaling in patients with recurrent viral infections.

Although scientists have been trying to establish a clinical assay to evaluate TLR functions of cells in human peripheral blood [78,79,80], most assays are somehow limited. Ida et al. studied the TLR-ligand stimulated cytokine production of peripheral blood DC subsets in whole blood [80]. Yet, the frequency of cytokine-positive DCs in whole blood was found to be lower when compared to that in freshly isolated PBMC. TLR ligand neutralizing factor in serum could be the interference. Deering and Orange avoided the influence of soluble factors in whole blood by measuring TNF-α secretion in the supernatant of isolated PBMC after different TLR agonist stimulations [78]. Nevertheless, TLRs are differentially expressed on different subpopulations of PBMCs [55]. Thus, the detection of TLR-defect could have been missed by evaluating the cytokine level in the supernatant of the whole PBMC population.

Furthermore, since all TLRs can trigger NF-kB activation in the final pathways, TNF-α is mostly chosen to be the read-out parameter. However, previous assays mainly focused on TNF-α secretion in APCs only, without evaluating the antiviral responses in NK cells and lymphocytes [78,79,80]. NK cells, as mentioned in previous sections, are particularly important in innate anti-viral and anti-tumor defences. Therefore, we wanted to develop a practical multi-color flow cytometry-based assay to evaluate TLR functions in different subsets of PBMCs, especially focusing on TLR-induced IFN-γ production in NK cells, and its association with recurrent HSV-1 infections and risk of gastric cancer. To further find out potential underlying genetic susceptibility mechanisms, subjects were genotyped for common TLR SNPs.

2 Aims and objectives

Toll-like receptors play important roles in recognizing conserved structures of pathogens and endogenous danger signals. TLR-mediated inflammatory responses are essential in controlling viral or bacterial infections. Deficiency or over-reaction in the TLR pathway results in susceptibility to a wide variety of human diseases and sometimes a poor clinical outcome. Polymorphisms in TLR signaling have been reported, which suggest heterogeneity of TLR responses among different individuals. TLRs are also expressed differentially in human PBMCs. In order to detect potential defects in TLR pathways which might be associated with disease susceptibility, we wanted to develop a clinical assay to evaluate TLR responses of each cell subset of PBMC.

Natural killer cells are indispensible in herpes virus infections, and exert vigorous anti-tumor activity. It has been shown that NK cells can directly respond to TLR stimulations. Therefore, we evaluated TLR responses of NK cells and the inter-individual variation of these responses. We were interested in the roles of TLR responses of immune cells, especially NK cells, in two clinical scenarios: the susceptibility to recurrent orolabial HSV-1 reactivations, Herpes labialis (HL); and the susceptibility to gastric cancer.

The main objectives of our study are as follows:

- To establish multi-color flow cytometric-assays for the evaluation of TLR responses of different PBMC subsets and TLR responses of isolated NK cells
- To analyze the association of differential TLR responses and susceptibility to recurrent HL
- To analyze the association of functional TLR polymorphisms and susceptibility to gastric cancer
- To investigate potential molecular mechanisms of NK cell TLR-hyporesponsiveness

3 Materials and Methods

3.1 Subjects

3.1.1 Subjects for the study of susceptibility to recurrent herpes labials

Human peripheral blood was collected from 32 clinically immunocompetent volunteers, mean age 29.9±9.8years. 19 of the blood donors had a history of HL (2-4 times/ year), while the other 13 donors of similar age and sex had no history of HL. All blood donors were free of acute viral infections or HL at the time of evaluation. Serological evidence of previous HSV-1 infection was investigated by the Institute of Virology, Charité - Universitätsmedizin Berlin. 6 HSV-1-seronegative healthy donors were also recruited. 35 ml of fresh blood was drawn from each donor into heparin-containing BD Vacutainer tubes and used for functional studies. Additional blood donors were further collected for TLR3 SNP genotyping, including DNA samples collected from the Institute of Microbiology and Hygiene, Charite. A total number of 53 controls and 51 individuals with HL histories were genotyped. The research was approved by the Charité institutional ethic committee and informed consent was obtained from each participant.

3.1.2 Subjects for the study of susceptibility to gastric cancer

DNA samples of gastric cancer patients were extracted and genotyped for TLR1 I602S SNP by our collaborator, Dr. Lutz Hamann/Prof. Ralf Schumann (Institute for Microbiology and Hygiene, Charite, CCM).

The majority of the patients enrolled were treated at the Robert-Rössle Cancer Center, Charité University Medical Center, Berlin-Buch for histologically proven gastric adenocarcinoma following surgery. Exclusion criteria were previous gastric surgery, preoperative chemotherapy or unknown UICC stage. Patients were followed up in the institution’s outpatient clinic. DNA-testing was permitted by written consent including DNA-testing before surgery. A total of 291 patients were included and analyzed for TLR-1 and -2 polymorphisms. In addition, 35 Patients were recruited from an ongoing case-control study by three different departments of the Charité University Medical Center Berlin: A) The Department of General, Visceral, Vascular and Thoracic Surgery; Division

of Molecular Biology, Campus Charité Mitte (CCM); B) The Department of Medicine I, Gastroenterology, Rheumatology and Infectious Diseases, Campus Benjamin Franklin (CBF); C) The Department of Hematology and Oncology, Campus Virchow-Klinikum (CVK). As control group, 410 H. pylori-positive patients, proven by urease breath test or histology, were recruited. Samples were included if gastric adenocarcinoma was excluded by histology [81]. All patients had histologically proven gastric adenocarcinoma. The study was approved by the Ethics committee of the Charité.

3.2 Reagents

3.2.1 TLR agonists

The TLR ligands used in this study are listed below (Table.1), along with the optimal concentrations for inducing maximal TNF-α responses in APCs (monocytes, mDCs, pDC and B cells) and IFN-γ responses in NK and T cells. According to manufacturer's suggestions and the concentrations suggested in the literatures, Pam3Cys was titrated from10 ng-10 μg/ml, poly(I:C) was titrated from 100 ng-100 μg/ml, LPS was titrated from 10 ng-1 μg/ml, R-848 was titrated from 100 ng-10 μg/ml, and CpG was titrated from 1-5 μM.

Table. 1. TLR ligands used to stimulate cells in the study. Pam3CSK4 is an agonist for TLR1/2 heterodimer. Resiquimod stimulates both TLR7 and TLR8.

TLR Ligand	Cognate TLR	Concentration	Source
Pam3CSK4 (Pam3Cys)	1/2 dimer	100ng/ml (APCs) 1μg/ml (NK/T)	InvivoGen, San Diego, CA, USA
Poly(I:C)	3	50μg/ml	InvivoGen, San Diego, CA, USA
LPS from E.coli, S form	4	100ng/ml	ALEXIS Biochemicals
Resiquimod (R-848)	7 and 8	2μg/ml	ALEXIS Biochemicals
ODN M362 (CpG oligonucleotide type C)	9	2.5μM	InvivoGen, San Diego, CA, USA

3.2.2 Flow cytometry reagents

3.2.2.1 Antibodies for detecting intracellular cytokine production in APCs

Productions of TNF-α and IFN-α were detected in monocytes, mDC, pDC and B cells with the following monoclonal antibodies:

Extracellular staining: For up to $2x10^6$ cells in 100 µl volume, 10 µl of PE-conjugated BDCA-1 (clone AD5-8E7, Miltenyi Biotec, Germany), 10 µl of APC-conjugated BDCA-2 (clone AC144, Miltenyi Biotec, Germany), 15 µl of PerCP-conjugated CD14 (clone MφP9, Becton Dickinson, Heidelberg, Germany), and 5 µl of APC-Cy7-conjugated CD19 (clone SJ25C1, Becton Dickinson) were added. (PE: phycoerythrin, APC: allophycocyanin, PerCP: peridinin-chlorophyll-protein complex)

Intracellular staining: For up to $2x10^6$ cells/100 µl, 0.5 µl of FITC-conjugated TNF-α (clone MAb11, Becton Dickinson), 0.5 µl of PE-Cy7-conjugated TNF-α (clone MAb11, e-Bioscience), or 10 µl of FITC-conjugated IFN-α (clone MMHA-11, PBL Biomedical Laboratories) was added. (FITC: fluorescein isothiocyanate)

3.2.2.2 Antibodies for detecting intracellular cytokine production in NK cells and T cells

Productions of TNF-α and IFN-γ were detected in NK cells and T cells with the following antibodies:

Extracellular staining: For up to $2x10^6$ cells/100 µl, 10 µl of PerCP-conjugated CD3 (Clone SK7, Becton Dickinson), 5 µl of APC-conjugated CD8 (clone RPA-T8, Becton Dickinson), and 15 µl of PE-conjugated CD56 (clone B159, Becton Dickinson) were added.

Intracellular staining: For up to $2x10^6$ cells/100 µl, 15 µl of FITC-conjugated IFN-γ (clone 25723.11, Becton Dickinson) and 0.5 µl of PE-Cy7-conjugated TNF-α (clone MAb11, e-Bioscience) were added.

3.2.2.3 Antibodies for detecting levels of TLR expression

TLR expression levels of PBMCs of high and low responders to the respective TLR stimulation were evaluated via flow cytometry by using monoclonal antibodies against TLR1 (FITC-conjugated, clone GD2.F4, abcam), TLR2 (PE-coupled, clone TLR2.1, e-Bioscience), TLR3 (PE-labeled, clone TLR3.7, e-Bioscience), and TLR8 (PE-labeled, clone44C143, e-Bioscience). The same fluorochrome-labeled isotype-matched monoclonal antibodies were used as controls. The mean fluorescence intensities (MFI) of the TLR signals were compared to the MFI of the respective isotype controls.

3.2.2.4 Buffers for FACS staining

PBS+2% Flebogamma (washing buffer, to prevent unspecific binding), 1mM EDTA, BD FACS Lysing Solution, BD FACS Permeabilizing Solution (for intracellular staining)

3.2.3 Cytokines and mediums

a. Co-stimulatory cytokines for TLR stimulations of NK cells and T cells:
   rhIL-12 (1 ng/ml, R&D Systems) , rhIL-15 (1 ng/ml, R&D Systems)

b. Cytokines for generation of monocyte-derived dendritic cells:
   rhIL-4 (20 ng/ml, R&D Systems), GM-CSF (50 ng/ml, Leukine, Berlex)

c. Positive control for NK cell and T cell stimulation:
   PMA (phorbol myristate acetate): 100 ng/ml (Sigma, Deisenhofen, Germany)
   Ionomycin: 1 µg/ml (Sigma, Deisenhofen, Germany)

d. Chemicals to stop cytokine secretion from the cell:
   Brefeldin-A (BFA): 7.5 µg/ml (Sigma, Deisenhofen, Germany)
   Monensin: 2 mM (Sigma, Munich, Germany)

e. Cell culture medium:
   RPMI 1640 Medium (Biochrom AG, Germany), containing 10% heat-inactivated fetal bovine serum, 1 U/ml Penicillin / Streptomycin, and 2 mM L-alanyl-L-glutamine; all reagents have been tested for very low endotoxin existence by the company. The RPMI medium was used for ex vivo incubation of PBMCs.

f. Freezing medium:

RPMI 1640 Medium (Biochrom AG, Germany), containing 10% DMSO and 36% serum; 1 ml of the freezing medium was used to resuspend 10^7 cells. Freezing container (Nalgene Cryo Container) was used to store the ice-cold cells in -80 ℃ before transferring to the nitrogen tank.

3.2.4 Cell isolation reagents

Biocoll (Ficoll) separating solution (density 1.077 g/ml, Biochrom AG, Germany) was used after adding RosetteSep NK cell/monocyte enrichment cocktails or before the application of MACS NK cell isolation kit.

RosetteSep NK cell enrichment cocktail (StemCell Technologies): Containing antibodies directed against cell surface antigens on human non-NK cells (CD3, CD4, CD19, CD36, CD66b) and glycophorin A on red blood cells (RBCs). After centrifugation, the non-NKs along with RBCs were at the bottom of the tube.

RosestteSep monocyte enrichment cocktail (StemCell Technologies): unwanted cells were linked to antibody complexes recognizing CD2, CD3, CD8, CD19, CD56, CD66b and glycophorin A on RBCs.

NK isolation kit (MACS, Miltenyi Biotec): untouched NK cells were isolated from PBMCs by depleting magnetically-labeled non-NK cells (T cells, B cells, stem cells, DCs, monocytes, granulocytes, and erythroid cells).

3.2.5 HSV-1 viral lysate

The recombinant HSV-1 used in this assay was kindly provided by Yasushi Kawaguchi. The UV-inactivated HSV-1 viral lysate was produced by the Charite Institute of Virology (kindly provided by Dr. Martin J. Raftery and Prof. Günther Schönrich). HSV-1 (KOS strain) was propagated and titered on Vero cells. After 15 minutes of UV inactivation, the remaining viral activity was < 10 PFU/µl. HSV-1 viral lysate also contained Vero cell debris. The freeze-thaw lysate of Vero cells were used as the control lysate. Both the viral lysate and control lysate were titrated and 10 µg/ml was the optimal concentration for the activation of 2.5×10^5 NK cells.

3.2.6 Reagents for DNA extraction and PCR

a. DNA extraction kit: QIAamp DNA Blood Mini Kit (QIAGEN)

b. DNA was extracted from whole blood or frozen PBMC and the concentration was determined by measuring OD260 on a spectrometer. The purity of the DNA was confirmed by calculating the OD260/OD280 ratio.

c. PCR: Taq PCR kit (USB Corporation, Cleveland, OH USA); MgCl2, PCR buffer and Taq-Polymerase purchased from Applied Biosystems, Rodgau-Jügesheim.

d. Primers: Primers detecting TLR SNPs are described later in the section “melting curve analysis of TLR SNPs”

e. Gel: 2% Tris-acetate-EDTA /ethidium bromide agarose gels

3.2.7 Reagents for RNA isolation and RT-PCR

a. Total RNA isolation kit: Nucleospin RNA II extraction kit (Macherey-Nagel)

b. cDNA synthesis: Quanti Test reverse transcription kit (QIAGEN)

c. RT-PCR: TaqMan Universal PCR Master Mix (Applied Biosystems)

d. Primers and probes: Primers and probes for detecting IFN-γ and the house keeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) were kindly provided by Birgit Sawitzki (Institute of Medical Immunology, Charite, CCM). TLR3 TaqMan Gene Expression Assay (Applied Biosystems) was used to detect basal TLR3 mRNA expressions.

3.3 Methods

3.3.1 Cell isolation

3.3.1.1 Isolation of peripheral blood mononuclear cells

35 ml fresh blood was collected from blood donors. 20 ml of the blood was used for PBMC isolation, and the other 15 ml underwent NK cell isolation. PBMCs were separated by using Biocoll solution, centrifugation, and were resuspended in 1 ml RPMI culture Medium, at $2x10^6$ cells for TLR ligand stimulation.

3.3.1.2 Isolation of NK cells

NK cells were isolated by adding the RosetteSep NK cell enrichment cocktail (StemCell Technologies) to fresh blood. The antibody cocktail connected CD3, CD4, CD19, CD36 and CD66b, to glycophorin A on red blood cells. The blood-antibody-cocktail mixture then underwent the same Ficoll separation steps as PBMC isolation, and the unwanted cell populations were discarded with the red blood cells. Purified NK cells were resuspended in 1 ml RPMI medium, at 3 $x10^5$cells for TLR ligand stimulations. The contamination of monocytes and CD3+T cell in NK cell fractions was below 1% (n = 3 analyses). In some experiments, NK cells were also isolated by MACS NK cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany); the purity of NK cells was 95-97%.

3.3.1.3 Isolation of monocytes and generation of monocyte-derived dendritic cells

Monocytes in some individuals were also isolated by using RosestteSep monocyte enrichment cocktail (StemCell Technologies). DCs were further generated from isolated monocytes: enriched monocytes were cultured in 96 well flat-bottom plate for 7 days in complete RPMI medium with IL-4 (R&D Systems) 20 ng/ml and GM-CSF (Leukine, Berlex) 50 ng/ml. In co-incubation experiments with $1.5x10^5$ isolated NK cells, $1.5x10^5$ isolated monocytes or $1.5x10^4$ monocyte-derived DCs were added.

3.3.2 Toll-like receptor ligand stimulations

a. Detection of TLR ligand-induced TNF-α/ IFN-α production in monocytes, mDCs, pDCs, and B cells (Antigen presenting cell panel / APC panel):

- PBMCs ($2x10^6$cells/ ml) were treated with Pam3CSK4, Poly(I:C), LPS, Resiquimod, OND M362, and the ODN M362 control (Invivogen). 7.5 µg/ml brefeldin A was added together with or in some experiments 30 minutes after the addition of the above TLR ligands.
- Cells were then stimulated for 4 hours at 37℃ incubator.
- TNF-α / IFN-α secretion in monocytes, mDC, pDC and B cells was detected with the fluorescence-conjugated antibodies for flow cytometric analysis.

b. Detection of TLR ligand-induced IFN-γ production in T cells and NK cells (NK / T cell panel):

- PBMCs (2x106cells/ ml) were treated with rhIL-12 (1 ng/ml) alone or with TLR ligands Pam3CSK4, Poly(I:C), LPS, and Resiquimod for 24 hours.
- 7.5 µg/ml of brefeldin A (Sigma, Deisenhofen, Germany) was added for the final 6 hours of incubation.
- IFN-γ secretion in NK cells and T cells were detected with fluorescence-conjugated antibodies for flow cytometric analysis.

3.3.3 Flow cytometry analysis

3.3.3.1 Staining procedures for extracellular cell markers and intracellular cytokines

After TLR-ligand stimulations, cells were washed with PBS+2% Flebogamma. 1mM EDTA was added for 10 minutes at room temperature and washed before the addition of antibodies for extracellular staining. The staining of extracellular markers was performed on ice for 15 minutes. Cells were then washed and 1x BD Lysis buffer was added for the removal of RBCs. Afterwards, cells were permeabilized with 1x BD FACS Permeabilizing buffer and stained intracellularly with antibodies against cytokines for 30

minutes on ice. Finally, the cells were washed and prepared for flow-cytometric analysis.

3.3.3.2 Gating strategies

a. Detection of TLR ligand-induced TNF-α/ IFN-α production in monocytes, myeloid DCs, plasmacytoid DCs, and B cells: PBMCs were first gated according to the forward scatter and the sideward scatter. Then monocytes and B cells were identified as CD14+ CD19-cells and CD14-CD19+ cells. The mDCs and pDCs were identified as CD14-CD19-BDCA1+BDCA2- and CD14-CD19-BDCA1-BDCA2+ cells, respectively. The proportion of TNF-α-positive cells (%) as well as the geometric mean fluorescence intensity (MFI) of TNF-α in TNF-α-positive population were determined.

b. Detection of TLR ligand-induced IFN-γ production in T cells and NK cells: Lymphocyte-gate was first determined according to the forward scatter and the sideward scatter. Then T cells were identified as CD3+CD56- population, while NK cells were identified as CD3-CD56+ population. The NK cells were further divided into $CD56^{bright}$ and $CD56^{dim}$ subpopulations. The percentage of IFN-γ-positive cells and geometric MFI of IFN-γ staining in IFN-γ-positive cells were calculated. All data were acquired on BD FACSCanto II flow cytometer, and analyzed using BD FACSDiva software v6.0 and FlowJo software v8.8.6.

3.3.4 NK cell degranulation assay

As previously described by Penack et al. [82], the detection of the lytic granule membrane protein CD107a on the surface of NK cells represents degranulation and secretion of cytotoxic granules, and its expression showed a good correlation with NK cell cytotoxicity. Therefore, we have performed the CD107a assay on 4 individuals to determine the ability of their NK cells to degranulate.

Isolated NK cells were pre-incubated with 1 ng/ml of IL-12 or with medium only for 20 hours. 2.5 x 10^4 isolated NK cells were resuspended in 1ml RPMI medium. The NK-sensitive cell line K562 cells (derived from patient with CML in blast crisis, Braunschweig, Germany) were added to isolated NK cells in effectors / target (E/T)

ratios of 1:1 and 1:5, in a total volume of 1 ml. Negative controls in the experiments were NK cells without the addition of target cells, and positive controls were samples treated with PMA/ ionomycin (PAM 100ng/ ml, ionomycin 1 µg /ml; Sigma, Deisenhofen, Germany). 20 µl PE-conjugated anti-CD107a antibody (clone H4A3, Becton Dickinson) was added during the co-incubation of NK cells and K562 cells. After 1 hour, 20 µl of the secretion inhibitor 2 mM monensin (Sigma, Munich, Germany) was added. After another 5 hours, cells were washed and stained with PerCP-labeled CD3, APC-conjugated CD56 (Becton Dickinson), and LIVE/DEAD dead cell stain kit (invitrogen). CD107a expression on viable CD3-CD56+ NK cells was analyzed by flow cytometry.

3.3.5 Total RNA isolation

1 million NK cells purified by MACS human NK cell isolation kit (Miltenyi Biotec) were either unstimulated or stimulated with 1ng IL-12 and 50 µg/ml poly(I:C) in 1ml medium for 6 hours. Total RNA was isolated using the Nucleospin RNAII extraction kit (Macherey-Nagel). Briefly, Cells were washed with PBS and lysed in 350 µl lysis buffer (RA1) plus 3.5 µl β-mercaptoethanol. The cell lysate was vortexed vigorously and was frozen immediately in liquid nitrogen. Lysed cells were stored at -80 ℃ until more samples were collected. Further steps began with lysate filtration, homogenization with 70% ethanol, and DNA was binded to the column and was digested by rDNase at room temperature for 30 minutes. Afterwards, the column membrane was washed and RNA was eluted by 22 µl RNase-free H_2O, and stored in nuclease-free collection tube at -80 ℃.

3.3.6 cDNA synthesis

12 µl RNA (<1 µg) was reverse transcribed by using Quanti Tect reverse transcription kit (QIAGEN). Briefly, RNA was denatured at 65 ℃ for 5 minutes, and possible contaminating genomic DNA was further cleared by gDNA wipeout buffer at 42 ℃ for 10 minutes. RNAs were then mixed with reverse transcriptase, RT buffer, and RT primer mix. The reaction was performed at 42 ℃ for 30 minutes and the reverse transcriptase was inactivated at 95 ℃ for 3 minutes. The synthesized cDNA was stored at -20 ℃ or 4 ℃ for subsequent PCR reaction.

3.3.7 Quantitative Real-Time PCR

IFN-γ and TLR3 mRNA expressions were detected by qRT-PCR. The expression of the house-keeping gene HPRT was used as an endogenous control for normalization of the amount of sample RNA. The forward and reverse primer mix and TaqMan Probes for detecting IFN-γ and HPRT were kindly provided by Birgit Sawitzki (Institute for Medical Immunology, Charite, Berlin). Primers and probe for TLR3 was purchased from Applied Biosystems as TLR3 TaqMan Gene Expression Assay (amplifying exon 3-4). FAM and TAMRA were linked to the 5'end and the 3'end of the probe as reporter and quencher dyes, respectively. The TaqMan Universal PCR Master Mix (Applied Biosystems) were added to the primer and probe mixture, and together with 1 µl of cDNA resulted in a final reaction volume of 13 µl. The reaction mixture were placed in MicroAmp optical tubes and caps (Applied Biosystems), and after a short centrifugation, PCR was performed on 7500 Real-Time PCR System (Applied Biosystems). The PCR reaction started with 50 ℃ for 2 minutes, 95 ℃ for 10minutes, and followed by 40 cycles of 95 ℃ for 15 seconds and 60 ℃ for 1 minute. Data were analyzed by using the Sequence Detector System (SDS) software (Applied Biosystems) according to manufacturer's instructions. All real-time PCR assays were done in duplicates, and amplification of non-template controls and an intron control were included to exclude genomic DNA contaminations.

Relative IFN-γ mRNA expression level induced by IL-12+poly(I:C) treatment was calculated using the ΔΔCt method using HPRT as reference gene and unstimulated cells as baseline. Fold induction of IFN-γ after poly(I:C) treatment = $2^{-\Delta\Delta Ct}$. ΔΔCt = (Ct IFN-γ (stimulated) – Ct HPRT (stimulated))-(Ct IFN-γ (unstimulated) – Ct HPRT (unstimulated)).

TLR3 mRNA basal expression levels were compared between poly(I:C)-high responders and -low responders: TLR3 mRNA expression in donor A is x fold of the level in donor B, x = $2^{-\Delta\Delta Ct}$. ΔΔCt = (Ct TLR3 (donor A unstimulated) – Ct HPRT (donor A unstimulated))-(Ct TLR3 (donor B unstimulated) – Ct HPRT (donor B unstimulated)).

3.3.8 DNA extraction

Genomic DNA was extracted from whole blood using QIAamp Blood Mini Kit (QIAGEN). Briefly, 200 µl blood stored in EDTA tube at -80 ℃ was equilibrated to room temperature, mixed with protease and lysis buffer, and was incubated at 56 ℃ for 10 minutes. 100%

ethanol was added, and the mixture was spun through the column. The column membrane was washed and the DNA was eluted by 100 µl elution buffer (AE). The concentration of extracted DNA was determined by using NanoDrop spectrophotometer (Thermo Scientific).

3.3.9 Melting curve analysis of TLR SNPs

Genotyping of TLR single nucleotide polymorphism (SNP) was performed by using 50-100ng genomic DNA and fluorescence-labeled hybridization FRET probes, followed by melting curve analysis on a LightCycler (Roche Diagnostics, Mannheim, Germany) as described before [40,83].

The primers and hybridization probes were purchased from TIB MOLBIOL (Berlin, Germany). In principle, the 'sensor' probe covers the position of the single nucleotide variance, and the adjacent 'anchor' probe has a much higher melting temperature. When samples are steadily heated, the 'target-probe' hybrid melts dependent on the melting point of the 'sensor' probe. Melting of the hybrid causes a spatial separation of the dye molecules and a drop in fluorescence, which is detected by the LightCycler. A single nucleotide difference will result in a decrease of the melting temperature.

Primers and probes for detecting TLR1 I602S SNP (rs5743618) were: TLR-1 forward: tgtgactacccggaaagttataga, and TLR-1 reverse: cccagaaagaatcgtgcc; TLR-1 sensor: ccatgctggtgttggctgtgactgtg-FL, TLR-1 anchor: Red640-cctccctctgcatctacttggat-pH

Primers and probes for detecting TLR3 L412F SNP (rs3775291) were: TLR-3 forward: ggaagataatgatattccaggcat, and TLR-3 reverse: gcaaaggagttcctagtcagc; TLR-3 sensor: tcttggttaggttgaatatgtgtaag-FL, TLR-3 anchor: Red640-gagaatgagcaagtgatacaaatgtttcat-pH

3.3.10 In vitro UV irradiation of PBMCs

PBMCs were isolated via Ficoll separation and washed by PBS. $5x10^5$ cells were placed in 2 ml PBS per well of the 6-well plate. Half of the plate was covered by foil paper as 'non-UV-irradiated' controls. The plate was placed on top of a UV-illuminator,

which emits UV-light in the UVB range (312 nm) and provides 0.001J/cm^2/sec. Cells were pulse-irradiated for 20 x 1 second, and received in total 20mJ/ cm^2. Afterwards, both UV-irradiated and non-irradiated PBMCs were washed with PBS and placed in 1 ml cell culture medium/ tube. Cells were further stimulated with IL-12 + poly(I:C), or with IL-12 alone for 24 hours. IFN-γ production of NK cells was detected by intracellular staining and flow cytometry.

In order to avoid direct UV-irradiation of the NK cells, PBMCs were further separated in some experiments by MACS (Miltenyi Biotec) into NK and non-NK populations. The non-NK subsets were either UV-irradiated or un-irradiated, and were added back to isolated NK cells for poly(I:C) stimulation.

3.3.11 HSV-1 infection of purified NK cells

The HSV-1 used in this assay was kindly provided by Yasushi Kawaguchi [84]. It contains the marker gene venus green fluorescent protein (VGFP), which has been inserted into the HSV-1 Us3 gene. 8 x 10^4 purified NK cells were cultured in 250 µl culture medium/ well on a 96-well round bottom plate. NK cells were pre-activated by 500 IU/ml IL-2 and 15µg/ml PHA for 24 hours before infection. Afterwards, live HSV-1 was inoculated onto NK cells at a multiplicity of infection (MOI) of 25 for another 24 hours by Dr. Martin J. Raftery (AG Schönrich, Institute of Virology, Charite, CCM). Infected NK cells were detected by examining intracellular GFP signals using fluorescence microscope and flow cytometry.

3.3.12 HSV-1 viral lysate stimulation assay

HSV-1 viral lysate was UV-inactivated and titrated from 2 µg/ml to 100 µg/ml. The control lysate (uninfected Vero cells) was also titrated for the same concentration as the HSV-1 lysate. 10µg/ml was the optimal concentration for inducing maximal amount of IFN-γ production in T cells/ NK cells and the same concentration of the control lysate stimulated a low background of IFN-γ production in these cells.

Human PBMC (2x10^6cells/ ml) or isolated NK cells (3 x10^5 cells/ml) were stimulated with HSV-1 viral lysate or control lysate in the presence or absence of 1 ng/ml IL-12 for 24 h. BFA was added at the last 6 hours. IFN-γ secretions in the NK cells/ T cells were then

analyzed via flow cytometry.

3.3.13 Statistical analysis

a. Detection of specific TLR-ligand-induced cytokine production in different cell subsets in the human PBMC: Non-parametric test was chosen to analyze the data. Data of cytokine production in monocytes, mDCs, pDC, B cells and T cells were analyzed from 8 donors and data of TLR ligand-induced IFN-γ secretion in non-purified and purified NK cells were collected from 10 donors. TLR ligand-triggered response was compared to the unstimulated samples (paired data). Wilcoxon's signed-ranks test was applied for analyzing the difference between the cytokine production in each cell population in the presence or absence of each TLR-ligand.

b. Comparison of different levels of TLR-ligand-induced cytokine in cells of asymptomatic healthy subjects and individuals with recurrent herpes labialis: Mann-Whitney U tests were performed to determine significant differences of the TLR-responses between the two groups (independent samples).

c. Fisher's exact test and chi-square test were used to analyze differences of frequencies of TLR3 L412F/TLR1 I602S variant allele and proportions of TLR3/TLR1 genotypes between individuals with and without recurrent HL, respectively.

d. The non-parametric Spearman's test was applied for correlation studies.

All statistical tests were performed on GraphPad Prism v.5.

4 Results

Part I Development of multi-color flow-cytometric assay

4.1 Establishment of a multi-color flow cytometric assay to evaluate TLR responses in different PBMC subsets

4.1.1 Gating strategies

A multi-color flow cytometric assay was developed to evaluate TLR responses in different PBMC subsets. The assay was divided into two panels: the antigen-presenting cell panel (the APC panel) for detecting TLR-ligand-induced cytokines (TNF-α/ IFN-α) in monocytes, mDCs, pDCs, and B cells; and the NK cell and T cell panel (NK/T cell panel) for detecting TLR-ligand-induced cytokines (IFN-γ/ TNF-α) in NK cells and T cells. For detecting the APCs, PBMCs were first gated according to the forward and sideward scatters (Fig. 1A). Monocytes were defined as CD14+ CD19- cells, and B cells were gated on CD14-CD19+ cells. Cells negative for both CD14 and CD19 were further divided into BDCA-1+ cells and BDCA-2+ cells, and were defined as myeloid DCs and plasmacytoid DCs, respectively. For detecting NK and T cells, lymphocyte gate was first determined by forward and sideward scatters (Fig. 1B). T cells were detected by gating the lymphocytes on CD3+CD56- cells, while NK cells were defined as CD3-CD56+ cells. The subpopulation of NK cells which express a higher level of CD56 as detected by PE-conjugated CD56 antibody (BD Biosciences) was gated for the evaluation of the functions of $CD56^{bright}$ NK cells. In some experiments, T cells were further gated on CD8 to distinguish the CD8+ from the CD8- subsets. TLR-triggered cytokine- producing cells were detected by gating on specific cell surface markers against the intracellularly-stained cytokines (e.g. CD14+CD19-TNF-α+ cells represented TNF-α-producing monocytes, and CD3-CD56+IFN-γ+ cells represented IFN-γ-secreting NK cells).

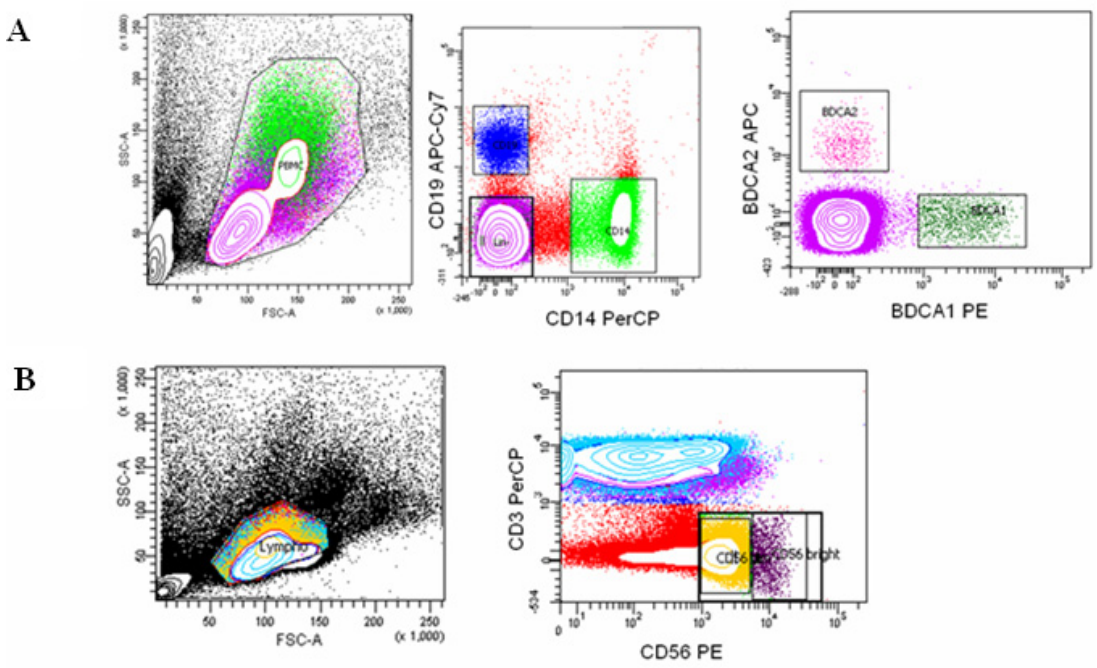


Fig. 1. Gating strategies. (A) Antigen-presenting cell panel. PBMCs were first gated, and divided into CD14+ (monocytes) and CD19+ (B cells). The double negative population was then gated on BDCA1 (marker for mDC) and BDCA2 (marker for pDC). **(B)** NK/T cell panel. CD3+ cells derived from the lymphocyte gate are T cells, and CD3-CD56+ population are NK cells, which were further divided into CD56bright (<10% of total NK cells) and dim subsets.

4.1.2 Concentrations of the TLR ligands were optimized and TNF-α and IFN- γ production were selected as the read-out of the assay

Using PBMCs, the TLR ligand concentrations were titrated in the range suggested by the manufacturers and previous studies. Pam3Cys was titrated from 10 ng-10 μg/ml, poly(I:C) was titrated from 100 ng-100 μg/ml, LPS was titrated from 10 ng-1 μg/ml, R-848 was titrated from 100 ng-10 μg/ml, and CpG was titrated from 1-5 μM. In case of the Pam3Cys-, LPS- and R-848- stimulation, the lowest concentration of the ligand to achieve the maximal TNF-α production in monocytes after 4 hours of incubation was selected as the optimal concentration for the assay (demonstrated in Table 1). Moreover, the optimal concentrations of poly(I:C) and CpG oligonucleotide were determined as the ones to induce the maximal cytokine production of myeloid DCs and plasmacytoid DCs, respectively. Both IFN-α and TNF-α secretions in pDCs were detected after 18 hours of R-848 and CpG ODN stimulations. However, the TNF-α response was more prominent than the IFN-α response (with a 4-fold higher response to CpG ODN and a 2-fold higher response to R-848), and could be detected in all cell

types of the APC panel 4 hours after all TLR-ligand stimulations. Therefore, TLR-ligand induced TNF-α production was determined as the final read-out of the APC panel of the assay.

Similarly, the TLR ligand concentrations in the NK/T cell panel were titrated. Instead of 4 hours, TLR agonists enhanced significantly the IFN-γ production in NK cells after 24 hours of stimulation. In the presence of 1 ng/ml IL-12, the fold of poly(I:C)-enhanced IFN-γ production in NK cells (12.5-fold) was greater than the fold of increased TNF-α (3.4-fold), compared to cells stimulated with IL-12 alone. Furthermore, IFN-γ secretion in NK and T cells represents an important effector function in antiviral defences. Therefore, IFN-γ was selected as the read-out for the NK/T cell panel.

4.1.3 IL-12 was added for IFN-γ profiling in NK cells and T cells

Although the NK cells and both CD8+ and CD8- T cells stimulated in the PBMC produced significant amount of IFN-γ in response to TLR-ligands (except to type C CpG and in some donors to Pam3Cys), isolated NK cells did not produce IFN-γ in response to any of the TLR ligands in the absence of co-stimulatory cytokines (Fig. 2). Therefore, for the profiling of IFN-γ production in NK cells, 1 ng/ml of IL-12 was added together with the TLR ligands for 24-hour stimulations.

After the addition of IL-12, we observed that all the TLR agonists, except type C ODN M362, enhanced substantially the IFN-γ production in isolated NK cells (Fig. 2). IL-12 also promoted the TLR-ligand-triggered IFN-γ response in non-purified NK cells. The response to poly(I:C) of NK cells in the PBMC increased almost 2-fold following IL-12 priming.

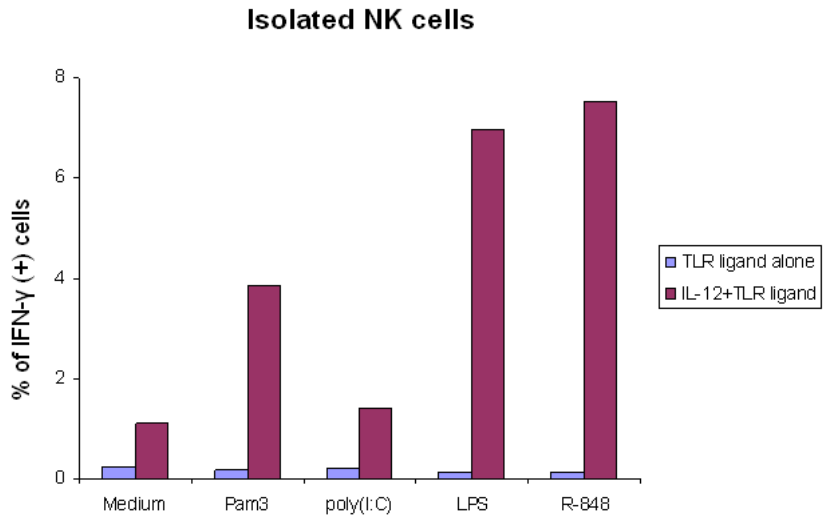


Fig. 2. TLR-ligand-stimulated response in isolated NK cells. The IFN-γ responses of isolated NK cells from one donor stimulated with TLR-ligand alone or with the addition of IL-12 for 24 hours are demonstrated as a representative. TLR-ligands triggered a substantial amount of IFN-γ production in isolated NK cells in the presence of 1 ng/ml IL-12.

4.1.4 NK cells and T cells could not be fully activated if BFA was added before TLR-ligand stimulation

Brefeldin-A (BFA) is known to block most of the cytokine secretions. In order to decrease the influence of cytokines secreted by bystander cells and at the same time measure the TLR response that is closest to *in vivo* biology, we added BFA to the whole PBMC population along with TLR ligands in some experiments. Instead of adding BFA 30-60 minutes after TLR stimulation in the antigen-presenting cell panel, BFA was added with TLR ligands at the beginning of incubation for 4 hours. Instead of adding BFA at the last 6 hours of TLR stimulation in the NK/T cell panel, PBMCs were first primed by IL-12 for 6 hours, and stimulated with TLR agonists in the presence of BFA for another 18 hours.

In monocytes, mDCs, pDCs, and B cells, the TNF-α responses to TLR ligands were similar when BFA was added immediately prior or half an hour later to TLR agonists (Fig. 3A). Interestingly, when BFA was added together with TLR stimulators, the responses of NK cells to TLR ligands were much lower (Fig. 3B) and the responses in T cells were completely abrogated (Fig. 3C).

Taken together, the results suggest that although TLR-ligands can stimulate the IFN-γ

production in NK cells and T cells in the presence of IL-12, other cytokines or direct accessory cell-contact signals are required to fully activate the TLR-induced IFN-γ responses of NK cells, and especially of T cells. However, the antigen-presenting cells produced TNF-α directly after TLR-ligand stimulation, showing that these cells are independent of autocrine/ paracrine signals produced by the PBMC populations in response to TLR activation.

Therefore, in our assay, we have decided to add BFA together with TLR ligands for APC panel, and to add BFA at the final 6 hours of 24-hour TLR-ligand stimulation in NK/T cell panel.

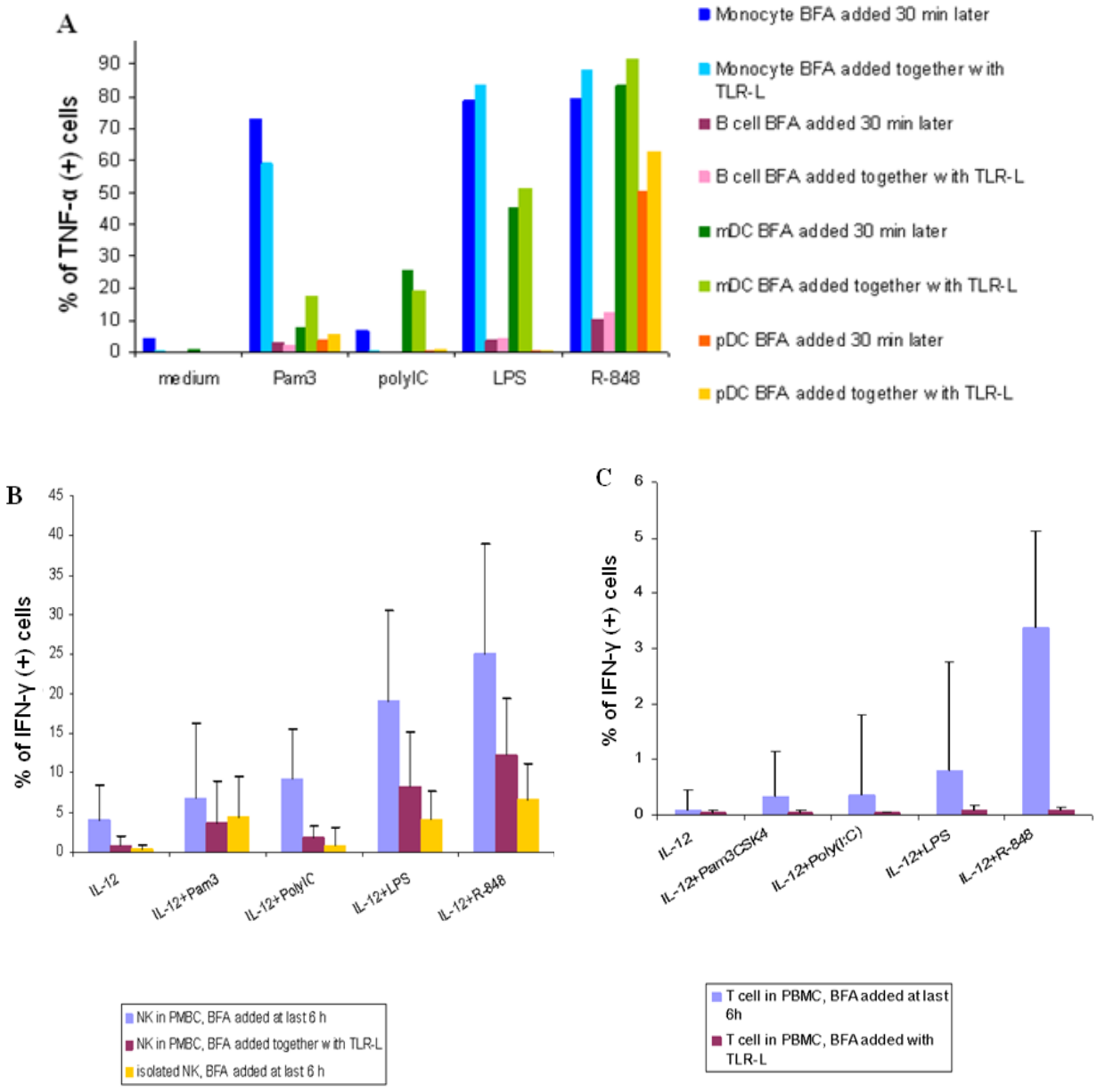


Fig. 3. BFA added together with TLR-ligands decreased bystander cell co-stimulations. BFA was added directly with TLR-ligands in these experiments. **(A)** Compared to the TLR-induced cytokine response when BFA was added 30 minutes later, the responses in the APC panel when BFA was added together with the TLR ligands were similar (the result shown here is one representative of 3 donors). **(B)** The response of NK cells detected in the PBMCs when BFA was added together with TLR-ligands was decreased (n = 4), as compared to the response when BFA was added at last 6 hours (n = 14), although the level of response was still higher than the response of isolated NK cells (n = 14), except the response to Pam3CSK4. The bar chart represents mean ± SD. **(C)** The response in T cells when BFA was added with TLR-ligands was almost not detectable (n = 5, mean ± SD), as compared with responses of T cells when BFA was added at the last 6 hours (n = 5, mean ± SD).

4.1.5 Effect of cell cryopreservation

Next, we sought to validate the assay for cryopreserved cells, since it could be applied to more clinical specimens that were properly cryopreserved. Fresh blood was taken from the same individual, and part of it underwent cryopreservation, the other part was stimulated directly with TLR-ligands. The cryopreserved PBMCs were kept at least one day in the nitrogen tank, and were thawed immediately on the day of experiment. The thawed cells were then rested overnight in the 37 ° C incubator and stimulated with the TLR agonists on the next day. The cryopreserved monocytes produced a similar amount of TNF-α in response to TLR-ligand stimulations as the freshly prepared monocytes (Fig. 4A); however, the cryopreserved NK cells produced less than half the amount of IFN-γ in response to TLR stimulations as compared to fresh NK cells (Fig. 4B). Therefore, we decided to work with fresh blood in our assay.

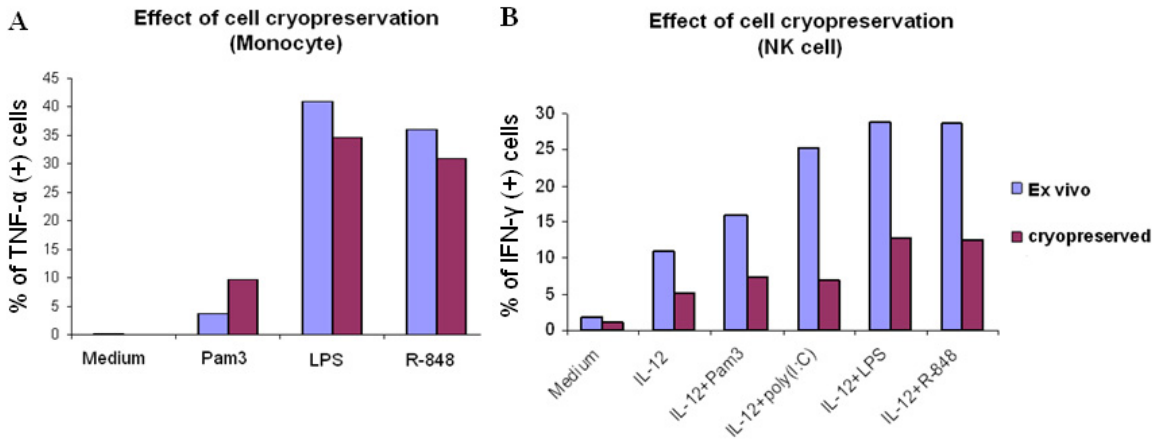


Fig. 4. Effect of cryopreservation in monocytes (A) and NK cells (B). TLR-ligand-induced cytokine productions of cells isolated from fresh blood (*ex vivo*) were compared with the TLR responses of cryopreserved cells from the same donor. One representative of 2 experiments is shown.

4.2 TLR-ligand-induced cytokine production in different cell subsets detected by the assay is in accordance with the literatures

4.2.1 TLR-ligand-induced TNF-α response in monocytes, mDCs, pDCs and B cells

To assess the biological specificity of TLR ligands and their capability to modulate TNF-α secretion in antigen-presenting cells and B cells, the frequency (%) of TNF-α-producing cells after different TLR ligand-stimulations was compared to the background frequency of TNF-α production in cells incubated with medium only (Fig. 5A). In accordance with the TLR expression in each cell type [55,61], Pam3CSK4 (TLR2/1 agonist) stimulated TNF-α responses mainly in monocytes and myeloid DCs (mDCs), and LPS stimulated significant TNF-α secretion in monocytes, mDCs, and B cells. Similarly, TLR3 agonist poly(I:C) induced a large amount of TNF-α in mDCs as previously reported [85]. R-848, a ligand for TLR7 and 8, triggered the biggest TNF-α production in every cell population. And consistently, ODN M362, a type C oligonucleotide containing unmethylated CpG motif, showed the specific ability to stimulate TNF-α secretion in pDCs and B cells. However, although isolated human pDCs do not express TLR2 and TLR4, considerable amounts of TNF-α responses were observed in pDCs within the PBMC after Pam3CSK4 (5.96%±5.48%) and LPS (1.98%±1.24%) stimulations, probably due to cell cross talks.

4.2.2 TLR-ligand-induced IFN-γ production in non-purified NK cells and T cells

We evaluated TLR-ligand-induced IFN-γ productions in NK cells and T cells by stimulating PBMCs with TLR1-8 ligands in the presence of 1 ng/ml of IL-12. The frequency of IFN-γ positive cells was compared to samples treated with IL-12 alone (Fig. 5B). Because antigen-presenting cells can provide signals to NK cells and T cells via soluble factors or cell contact [86,87], we investigated the NK cell response to TLR ligands in both purified and un-separated populations.

Poly(I:C), LPS, and R-848 stimulations significantly enhanced IFN-γ secretion of NK cells and T cells, as compared to responses triggered by IL-12 only (Fig. 5B, Wilcoxon's signed rank test, all $P < 0.05$). Isolated NK cells also produced substantial amounts of IFN-γ in response to these TLR agonists (Fig. 5B). Responses of isolated NK cells will

be described in the next section in detail.

Interestingly, we observed Pam3Cys-hyporesponsiveness in non-purified NK cells of 5 donors (Fig. 6). Monocytes and myeloid DCs (mDCs) detected in the PBMC of these 5 donors, however, showed enhanced TNF-α production to Pam3Cys stimulations (Fig. 6). These results suggest an inhibitory effect of PBMC population on NK cells in these donors. In order to avoid this inhibitory effect and also to minimize the bystander effect of accessory cells, we focused on the TLR responses of isolated NK cells.

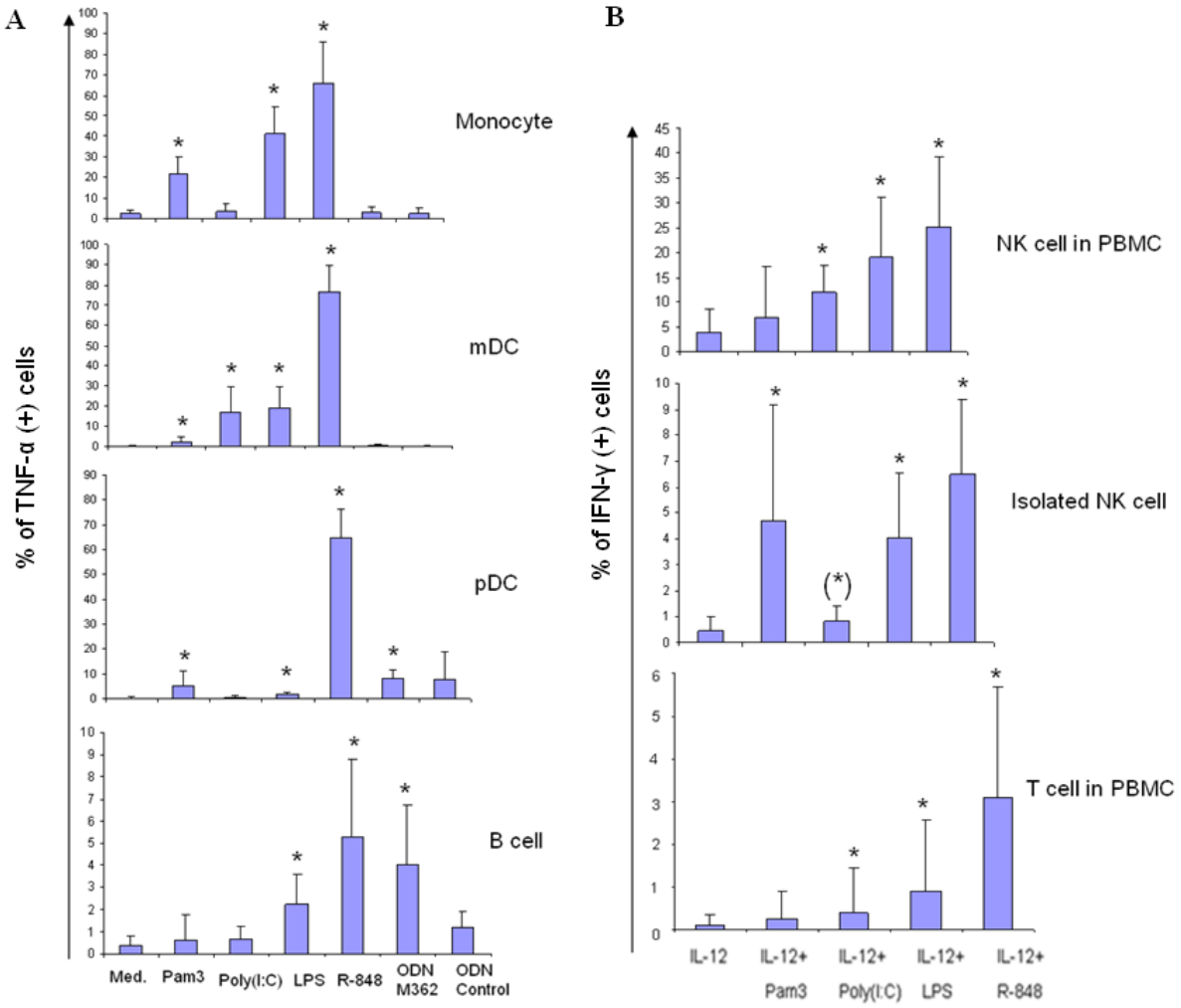


Fig. 5. Specific responses to TLR stimulations in different cell subsets of human PBMC. (A) TLR-induced TNF-α production in APC panel, n = 8, the bar chart represents mean ± SD. **(B)** TLR-induced IFN-γ production in NK/T cell panel, n = 10, the bar chart represents mean ± SD. ∗: Wilcoxon's signed rank test, two-tailed $P < 0.05$; (∗): one-tailed $P < 0.05$

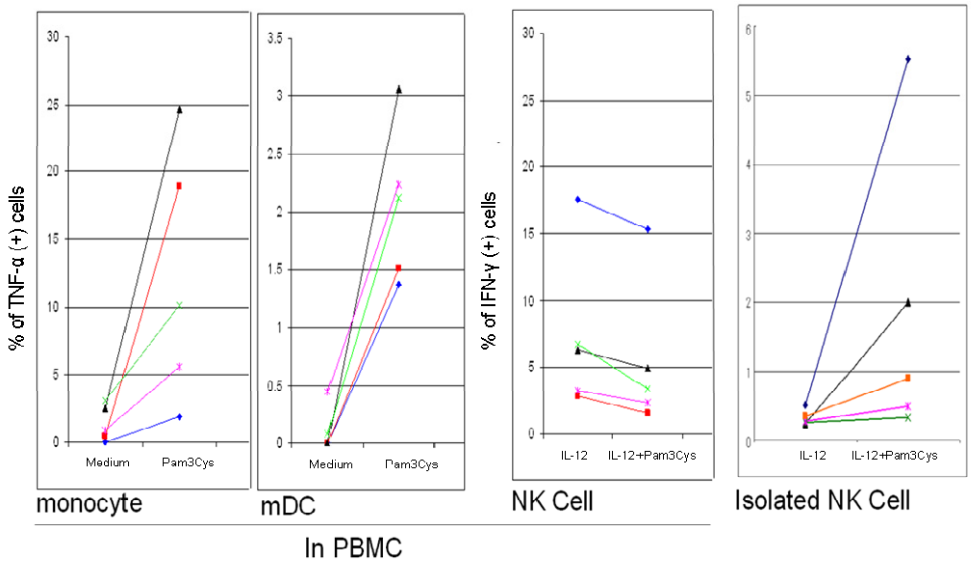


Fig. 6. Hyporesponsiveness to Pam3CSK4 of NK cells in PBMCs of 5 donors. Pam3CSK4-induced responses of monocytes, myeloid DCs (mDCs), and NK cells detected in PBMC, and the response of isolated NK cells in 5 donors are demonstrated. Each symbol represents a single donor.

4.2.3 TLR responses in isolated NK cells

At this point we have optimized the conditions of our assay for the evaluation of TLR responses in different subsets of human PBMCs. Since NK cells are critical components of HSV-1 infections, we further examined specific TLR ligand-induced responses in isolated NK cells in detail.

As described in the previous paragraphs, TLR agonists triggered negligible IFN-γ secretion in freshly isolated NK cells without the addition of co-stimulatory cytokines. It has been reported that IL-12 has synergistic effects with TLR agonists and can promote IFN-γ production in NK cells [57]. In accordance with the literature, 1 ng/ml IL-12 alone resulted in a minimal percentage of IFN-γ-producing NK cells (median 0.5%, range 0.2-1.9%, n=18); while the addition of TLR2/1 agonist Pam3Cys, TLR3 agonist poly(I:C), TLR4 agonist LPS, and TLR7/8 agonist R-848 all enhanced significantly the number of IFN-γ secreting NK cells (Fig. 8A-B). The TLR9 agonist ODN M362 (invivoGen), however, did not trigger IFN-γ response in isolated NK cells either in the presence or absence of IL-12 (data not shown).

We further compared TLR responses in the CD56 dim (median 96.3%, range 92.7%-98.6%) and bright (median 3.7%, range 1.4%-7.3%) subpopulations of NK cells. Although being a minor subset, the frequency of IFN-γ-producing cells was much higher

in $CD56^{bright}$ NK cells: with median 11.3-fold, 1.5-fold, 10.2-fold, and 21.4-fold increase in response to Pam3Cys, poly (I:C), LPS and R-848, respectively, as compared with $CD56^{bright}$ NK cells stimulated with IL-12 alone, n=18. Representative FACS graphs of TLR-triggered IFN-γ responses in $CD56^{bright}$ NK subset are shown in Fig. 8A. Frequencies of IFN-γ-producing cells in response to TLR-ligands were compared between NK cell subsets in Fig. 8B.

In addition, we observed considerable inter-individual variations in the responses to TLR stimulations, ranging from 1.3-fold to 26.3-fold increase (Pam3Cys), 0.6-fold to 7.0-fold increase (polyI:C), 0.7 fold to 27.7 fold increase (LPS), and 1.9 fold to 36.9 fold increase (R-848) of IFN-γ-producing NK cells. Furthermore, TLR responses were heterogeneous in several subjects, who showed high IFN-γ responses to some TLR ligands and low IFN-γ responses to other TLR agonists (data not shown). The magnitude of response was, however, highly reproducible. In 6 subjects a second sample was analyzed on a different date (over one month period), showing a high correlation (r_s= 0.908, P<0.001) (Fig. 9).

Collectively, these results demonstrate that IL-12-preactived NK cells respond to TLR ligands and that the magnitude of this response varies between individuals.

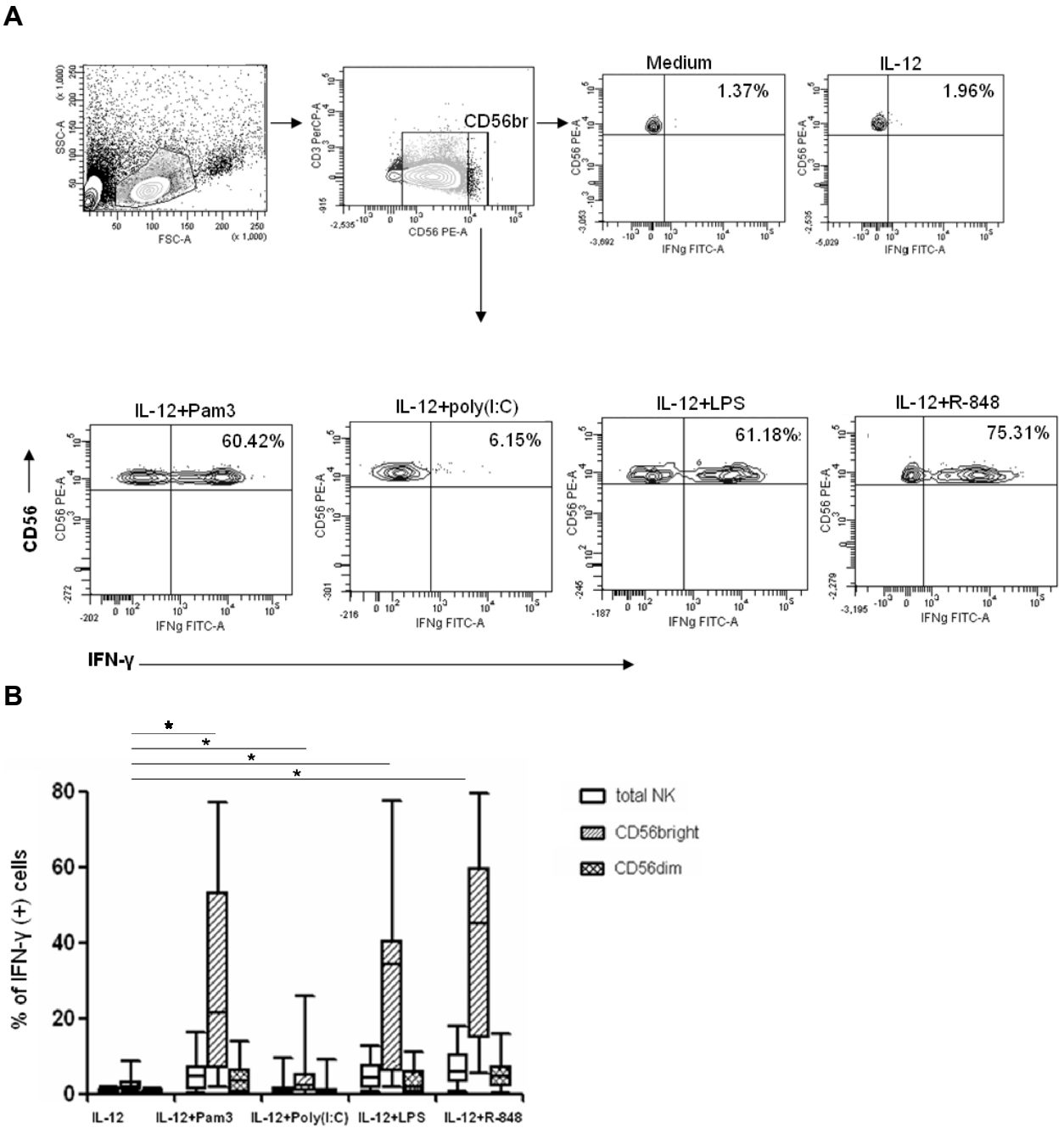


Fig. 8. TLR2/1-, TLR3-, TLR4-, and TLR7/8-agonist-induced IFN-γ response in isolated human NK cells activated with IL-12 was analyzed by flow cytometry. A: PBMCs were stimulated with IL-12 or IL-12 plus TLR agonists for 24 hours. An example of the gating strategy is shown; the percentages of $CD56^{bright}$ NK cells producing IFN-γ are shown in the upper right quadrant of each plot. **B:** Higher frequencies of TLR ligand-induced IFN-γ production were observed in the $CD56^{bright}$ NK cell subset. Box-whisker plot represents median, minimum, and maximum frequencies of IFN-γ positive cells from 18 donors. Wilcoxon's signed rank test was performed for detecting differences of NK cell responses to IL-12 alone and to IL-12+TLR-ligand, *: $P < 0.05$

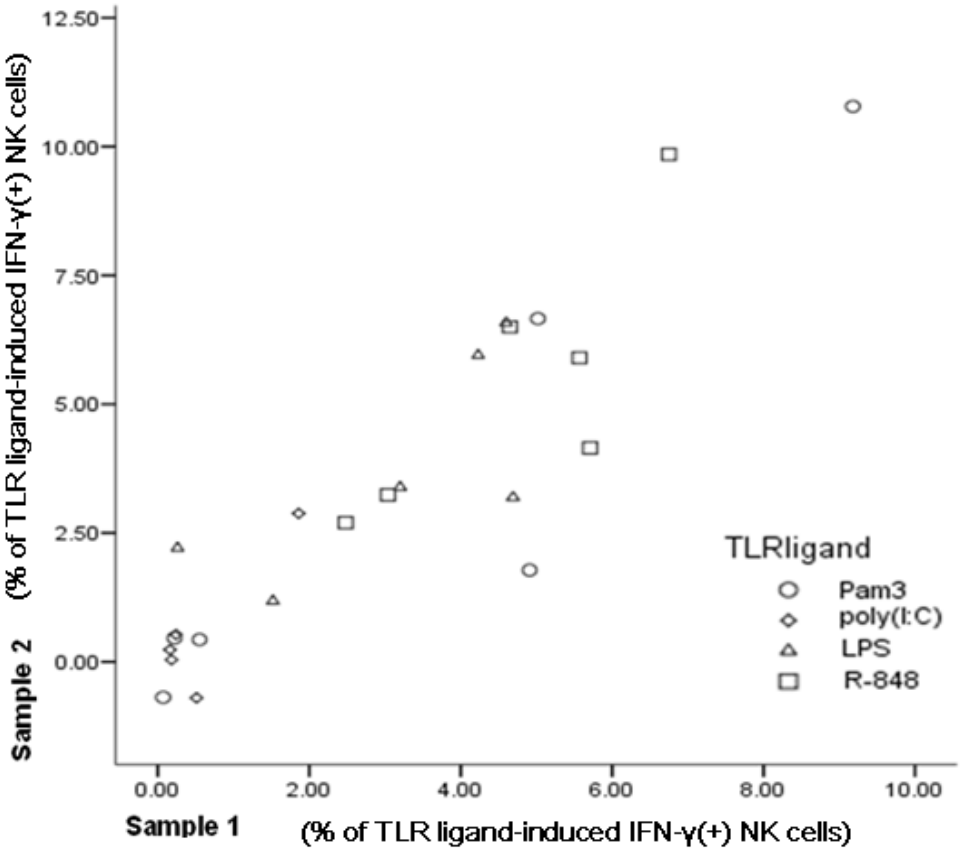


Fig. 9. The magnitude of TLR-agonist-induced response in NK cells from 6 donors was highly reproducible. The assay was performed twice for each of these donors on two different dates. Each dot represents a result measured at the same time point on date 1 or date 2, and different symbols indicate NK cell responses to different TLR ligands. A good correlation ($r = 0.908$, $P < 0.001$) is shown.

Part II TLR responses in subjects with recurrent HL

4.3 Detection of TLR responses in different PBMC subsets of subjects with HL

In the second part of my project, TLR responses in subsets of PBMCs were examined in people with histories of recurrent HL.

4.3.1 The amounts of TLR ligand-induced cytokines detected in unfractionated APCs, NK cells and CD8+T cells were not different between HL subjects and asymptomatic controls

TLR ligand-induced TNF-α production in monocytes, myeloid DCs, plasmacytoid DCs, and B cells were compared within the PBMC population of asymptomatic healthy controls (n= 8) and HL subjects (n= 9) (Fig. 10). Similarly, IFN-γ responses triggered by TLR1-8 agonists in NK cells and CD8+T cells were also evaluated in the 2 groups. No statistically significant difference in the frequency of cytokine-producing cells was found.

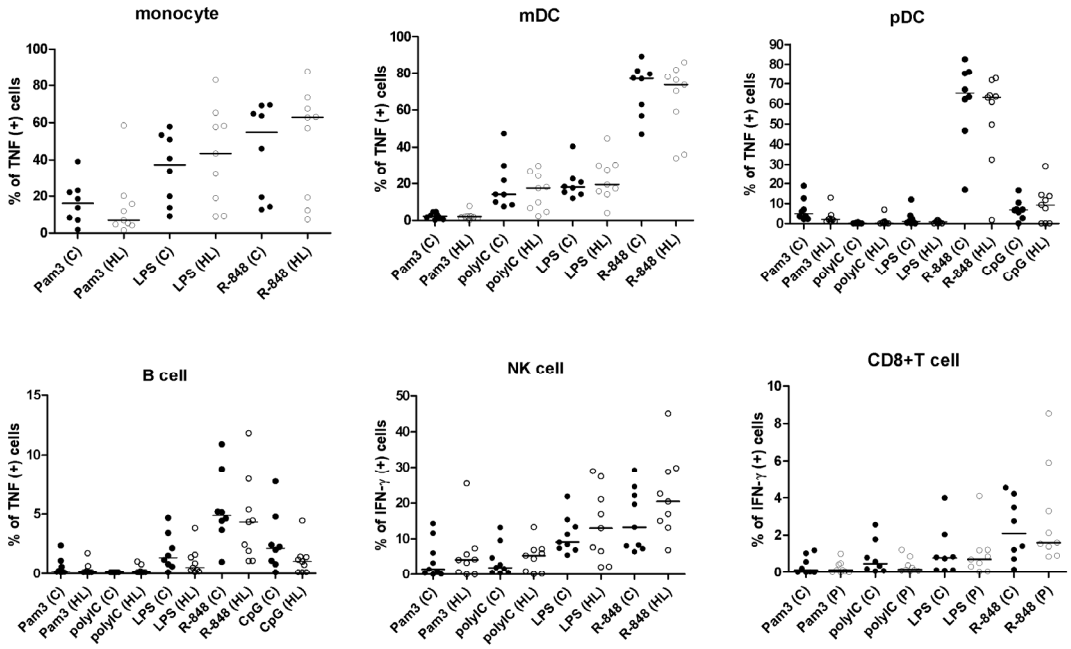


Fig. 10. TLR agonist-induced IFN-γ secretion in PBMC subsets of people with recurrent HL and of controls. The herpes labialis group is designated as (HL), open circles; n=9. Group of asymptomatic healthy controls is designated as (C), closed circles; n=8. The background production of TNF-α in unstimulated sample of the APC panel had been subtracted. The background secretion of IFN-γ in response to IL-12 had also been subtracted in the NK/T panel. Lines represent medians. Mann-Whitney U test showed no significant difference of TLR responses of PBMC subsets between the two groups.

4.3.2 Poly(I:C)-triggered responses of purified NK cells are significantly lower in subjects with HL

To study if differences in purified NK cell responsiveness to TLR ligands might correlate with susceptibility to HL, NK cells were isolated and stimulated with IL-12 plus agonists for TLR2/1, 3, and 7/8, which are the TLRs known to be involved in HSV infections (Fig 11A-11C). Remarkably, the frequency of IFN-γ-producing purified NK cells in response to the TLR3 agonist poly(I:C) was significantly reduced in the HL group (median, HL vs. control: 0.5% vs. 1.4%, $P<0.01$), while responses to agonists of TLR2/1 (Pam3Cys) and TLR7/8 (R-848) were not different (Fig. 11B-C). Cytometric bead array further detected decreased IFN-γ production in the supernatant of poly(I:C)-treated isolated NK cells, (median 147.7 pg/ml vs. 839.2 pg/ml, $P = 0.047$) (Fig. 11D). Total RNAs of purified NK cells derived from 3 poly(I:C)-high responders in the control group and from 3 donors of the HL group (low responders) were extracted after stimulation with poly(I:C) for 6 h. Quantitative real-time- PCR (qRT-PCR) showed that the induction of IFN-γ mRNA in HL subjects is also impaired (median 165.4 fold vs. 18.8 fold, $P = 0.029$)(Fig. 11E). Taken together, we have detected reduced poly(I:C)-induced IFN-γ secretion in isolated NK cells of people with recurrent HL, both at the protein and mRNA levels.

In order to exclude the possibility that the observed TLR-hyporesponsiveness is merely due to an enhancement of a subtle defect in the responsiveness to IL-12 alone, we have compared the IFN-γ production of NK cells in controls and HLs after different doses of IL-12 stimulations. No significant difference was observed at the IL-12 concentration (1 ng/ml) we used for IFN-γ-profiling of NK cells in this study (Fig. 11B) or at a higher IL-12 concentration (10 ng/ml) (Fig. 11F). Moreover, when we stimulated the purified NK cells derived from both groups with PMA/Ionomycin, no difference was detected in the level of IFN-γ responses (Fig. 11G), indicating that there is no obvious general defect in the ability of NK cells from HL subjects to produce IFN-γ.

Therefore, our data suggest that the observed poly(I:C)-hyporesponsiveness of purified NK cells from HL subjects might be due to a specific defect in NK cells involving TLR3 signaling.

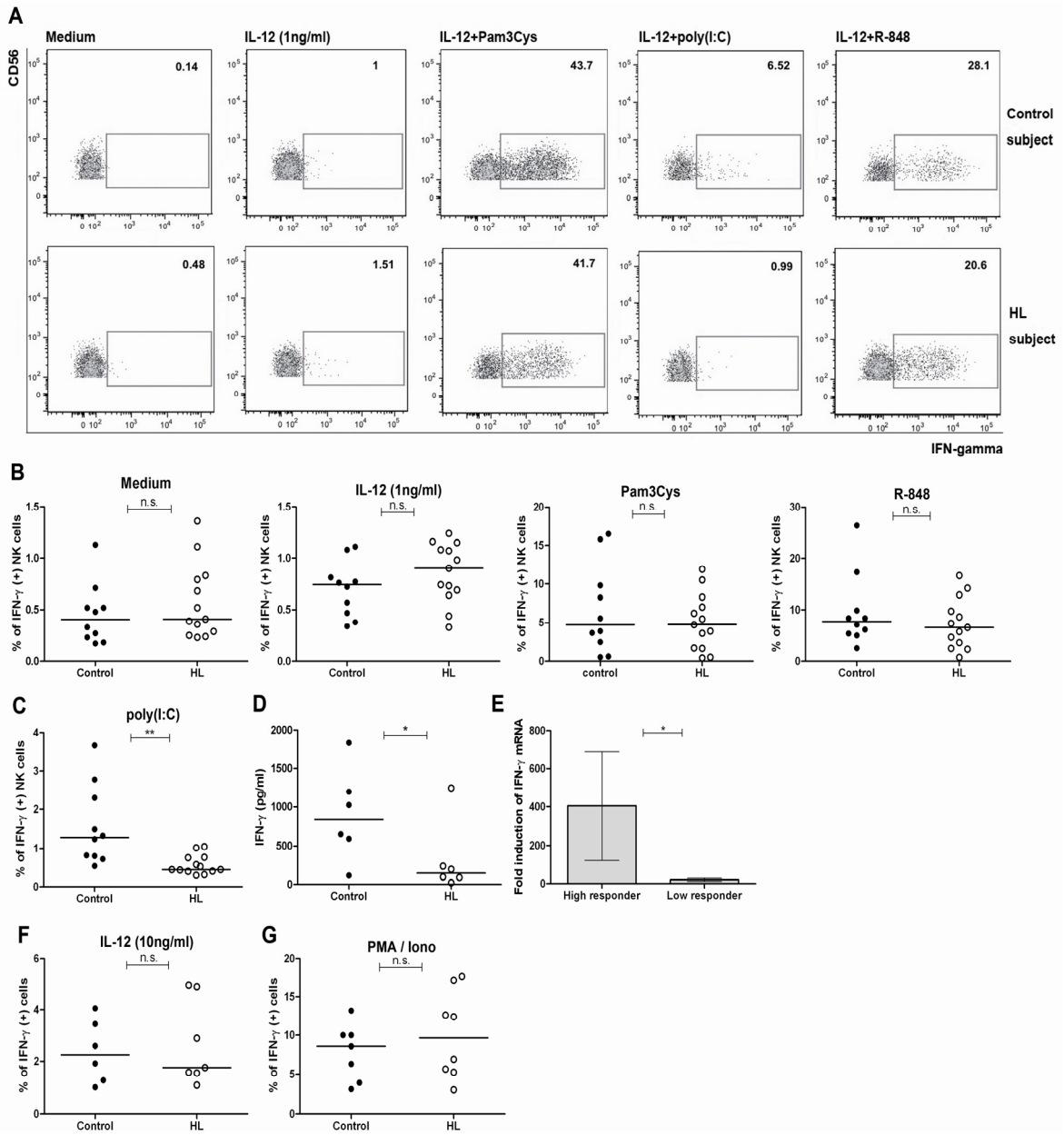


Fig. 11. Poly(I:C)-induced IFN-γ responses in isolated NK cells of HL individuals are lower than controls. (A) Purified NK cells were stimulated with TLR agonists in the presence of 1 ng/ml IL-12 for 24 h. Representative FACS graphs of IFN-γ-producing NK cells in response to different TLR agonists in one control and one HL subject are shown. Percentages of IFN-γ-positive cells are marked in the right upper corner of each graph. **(B-C)** IFN-γ responses in isolated NK cells of HSV-1 seropositive asymptomatic controls and HL subjects were compared. Control group: n=10, HL group: n= 13. Lines represent medians. **P<0.01 by Mann-Whitney U test. n.s.: P>0.05, not significant. **(D)** Amount of IFN-γ detected in supernatant of isolated NK cells by cytometric bead array after stimulation for 18 h with poly(I:C). Lines represent medians. *P<0.05 (E) Total mRNA was collected from purified NK cells stimulated with poly(I:C) for 6 h or left unstimulated. Fold induction of IFN-γ mRNA in 3 poly(I:C)-high and 3 poly(I:C)–low responders is demonstrated, using HPRT as calibrator. Error bars represent mean±SEM. (F) 10 ng/ml IL-12 and (G) 100 ng/ml PMA +1 μg/ml ionomycin induced similar levels of IFN-γ responses in isolated NK cells of controls and of HL subjects.

4.3.3 The percentage of NK cells is lower in people with recurrent HL

We next examined by flow cytometry the percentage of NK cell subsets within PBMCs of individuals with and without recurrent HL. Interestingly, people with HL showed significantly lower frequencies of NK cells in the lymphocyte population (median, HL vs. control: 12.1% vs. 27.9%, P = 0.019) (Fig. 12). We did not observe a difference in the frequencies of other cell subsets.

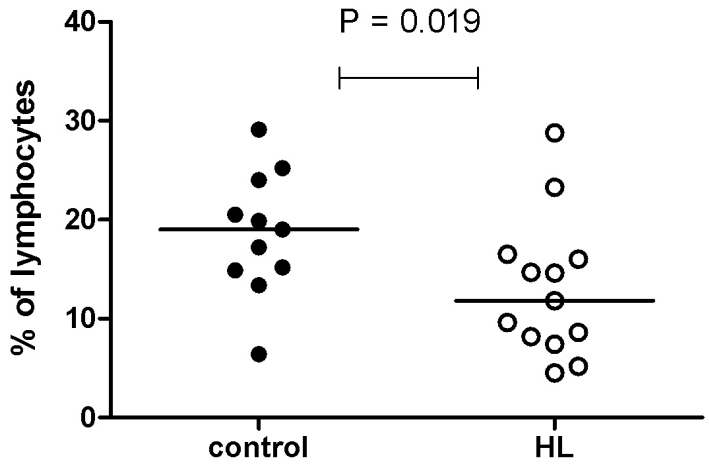


Fig. 12. Percentage of NK cells in lymphocytes of asymptomatic controls and individuals with HL. Lines indicate medians. Mann-Whitney U test was performed.

4.3.4 The co-stimulatory function of accessory cells restored the impaired poly(I:C) response of isolated NK cells

It has been reported in two HSE children with TLR3 mutation that although the isolated NK cells showed impaired response to poly(I:C), normal IFN-γ production of NK cells were observed when stimulated in the presence of APCs [26]. Similarly, we did not detect a difference in the NK cell response to poly(I:C) when we compared the IFN-γ secretion of NK cells in PBMCs of controls and of HL subjects (see section 4.3.1 and Fig. 13A). The data suggest that NK cell hyporesponsiveness to poly(I:C) could be restored by accessory cells in the PBMCs.

To further study if the co-stimulatory effect is mediated by cell-cell contact or soluble factors, we conducted co-incubation experiments on two HL subjects who had impaired IFN-γ responses to poly(I:C) in their purified NK cells, but high poly(I:C)-triggered

responses in the unfractionated NK cells. NK cells isolated from each donor were incubated alone or with the addition of autologous monocytes, monocyte-derived DCs, or supernatant collected from poly(I:C)-stimulated PBMCs. We observed that the lowered poly(I:C) response of NK cells could be compensated in one donor by DCs and in the other by monocytes, while the supernatant had no effect (Fig. 13B). These results indicate that co-stimulatory functions of APCs (monocytes, DCs) play an important role in restoring the impaired IFN-γ response to poly(I:C) in NK cells of HL individuals.

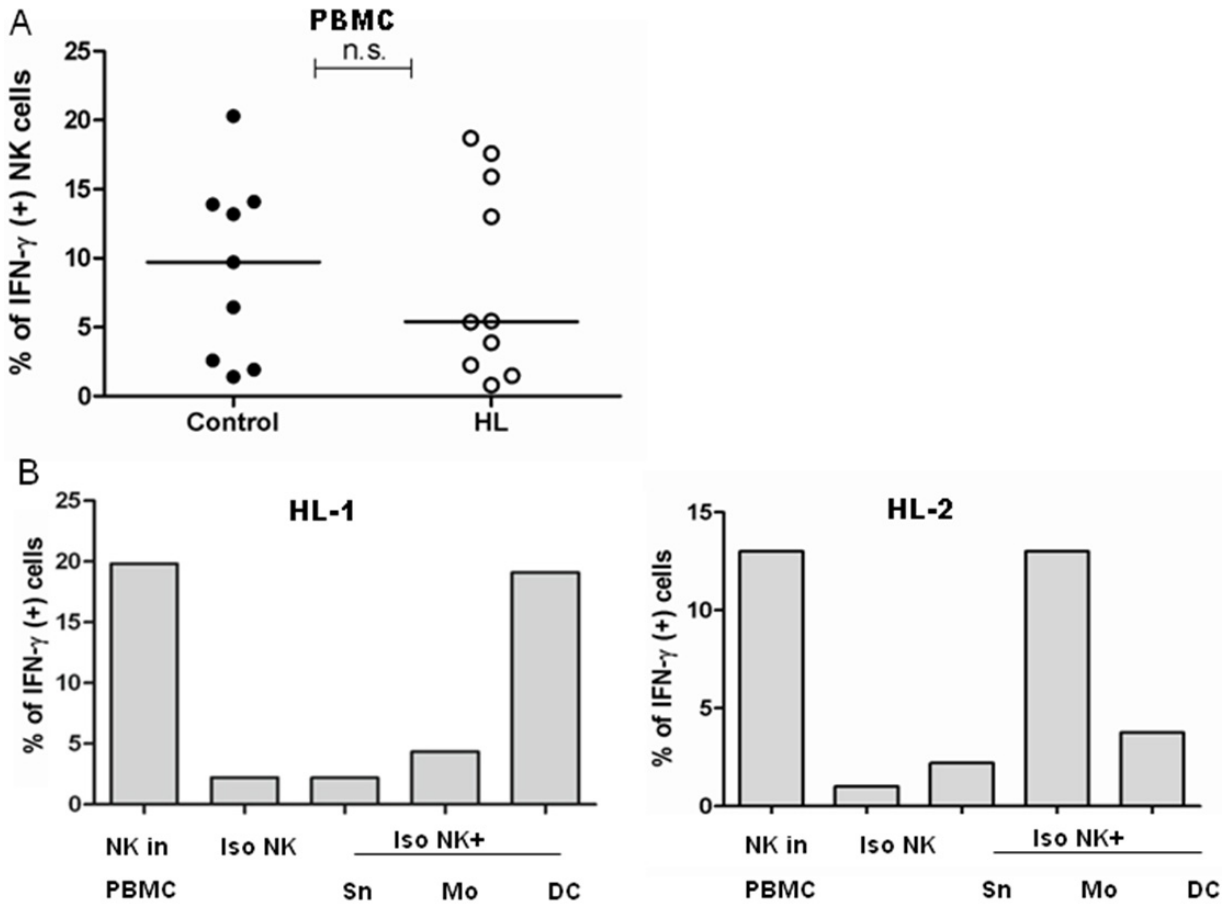


Fig. 13. The co-stimulatory function of APCs restored the impaired poly(I:C)-responsiveness of isolated NK cells in some people with HL. (A) poly(I:C)-induced NK cell IFN-γ responses detected in the PBMCs of the control and of the HL subjects are shown. Lines represent medians. **(B)** Isolated NK cells derived from 2 HL individuals (HL-1, HL-2), who showed high IFN-γ responses in PBMCs, were co-incubated with supernatant of poly(I:C)-stimulated PBMCs (Sn), with autologous monocytes (Mo), or with monocyte-derived DCs during 24-hour poly(I:C)-stimulations. % of IFN-γ-positive NK cells is demonstrated.

4.4 Mechanisms of poly(I:C)-hyporesponsiveness in NK cells

4.4.1 The amount of intracellular TLR3 expression did not correlate with the level of poly(I:C)-responsiveness in NK cells

Differential NK cell responsiveness to poly (I:C) has been suggested to be associated with the heterogeneity of TLR3 mRNA transcripts [88]. In order to find out if the ability to respond to poly(I:C) stimulation is correlated with the level of TLR3 in resting NK cells, we evaluated the amount of TLR3 via flow cytometry. A TLR3 monoclonal antibody (cloneTLR3-7, e-Bioscience) and its isotype control were used. Representative flow-cytometric graphs of TLR3-staining of NK cells are shown in Fig. 14A. While almost all NK cells are positive for intracellular detection of TLR3, only a small subset of NK cells express TLR3 on the cell surface. Therefore, we measured the mean fluorescence intensity (MFI) of intracellular TLR3, and evaluated the percentage of surface TLR3-positive NK cells.

Unlike the previous report, there was no difference in the level of total TLR3 protein detected in NK cells derived from 7 poly(I:C)-high and 7 poly(I:C) –low responders (Fig. 14B, left panel). The quantity of TLR3 mRNA in resting purified NK cells was also compared to the fold induction of IFN-γ mRNA after a 6-hour treatment of poly(I:C) via qRT-PCR, using HPRT as an endogenous control. No correlation was found between the mRNA levels of TLR3 and poly(I:C)-induced IFN-γ in 7 individuals (r_s=-0.32, P=0.5). We further compared the surface expression of TLR3 in 6 poly(I:C)-high and 6 poly(I:C)-low responders (Fig. 14B, right panel). Interestingly, three low responders had lower surface TLR3 expression on their NK cells.

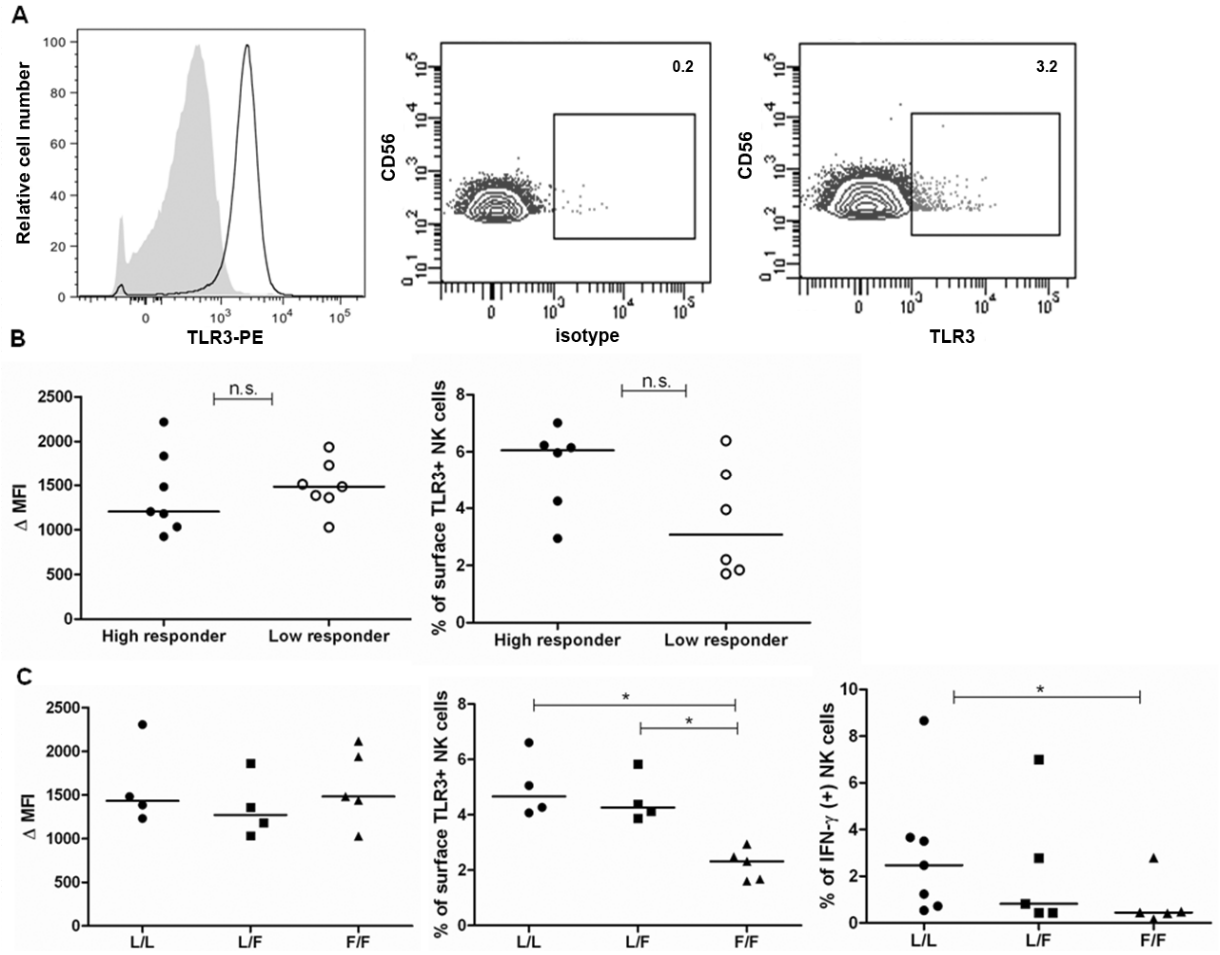


Fig. 14. Expression of TLR3 on NK cells and impairments of TLR3 surface expression and poly(I:C)-induced IFN-γ production in NK cells of people with homozygous TLR3 L412F genotype. **(A)** A representative histogram of intracellular TLR3 staining (shaded curve indicates isotype staining); and dot plots of extracellular TLR3/isotype staining of one donor are shown. **(B)** Left graph: The mean fluorescence intensities (MFIs) of intracellular TLR3 were detected in NK cells of 7 poly(I:C)-high and 7 poly(I:C)–low responders. ΔMFI: MFI of isotype control staining was subtracted from MFI of TLR staining. Right graph: The % of TLR3-surface-expressing NK cells in 6 poly(I:C)-high and 6 poly(I:C)–low responders. Lines represent medians. **(C)** Left graph: Intracellular TLR3 levels of people with different TLR3 L412F genotypes are similar. Lines represent medians. Middle graph: Percentage of TLR3-surface-expressing NK cells is lower in F/F genotype. *P<0.05 by Mann-Whitney U test. Right graph: Poly (I:C)-induced IFN-γ responses in isolated NK cells derived from donors with different TLR3 genotypes. *P<0.05.

4.4.2 TLR3 412 F/F impairs TLR3 surface expression and IFN-γ response to poly(I:C)

The TLR3 L412F genetic polymorphism (rs3775291, Ex4+601C>T) has a high allele frequency in Europeans and Asians (~30%). This variant has been reported not to affect

the total TLR3 expression level, but can lower the poly(I:C)-mediated NF-κB activity in reporter assays [89]. The authors also showed that 293T cells transfected with L412F had lower surface expression compared to wild type [89].

To characterize the functional impact of TLR3 L412F SNP on NK cells, we evaluated the TLR3 expression and response to poly(I:C) in subjects with different genotypes. There is no difference detected in the intracellular level of TLR3 (Fig. 14C, left panel). However, the percentages of TLR3-surface-expressing NK cells derived from individuals homozygous for the TLR3 L412F SNP (412F/F) were significantly lower than those derived from 412F/L and 412L/L individuals (median 2.5% vs. 4.3%, P= 0.01; Fig. 14C, middle panel). In line with the expression data, the poly(I:C)-induced IFN-γ production in purified TLR3 412F/F NK cells was significantly decreased as compared to the IFN-γ response in wild type NK cells (median 0.5% vs. 2.5%, P= 0.03; Fig. 14C, right panel).

To study if there is an association of HL with TLR3 L412F polymorphism, we genotyped subjects with and without histories of recurrent HL for TLR3 L412F by PCR and subsequent melting curve analysis. Of the 51 HL subjects and 53 controls genotyped in this study, we found a trend of higher mutant allele frequency in people with HL (38.2% vs. 28.3%, P= 0.08, Table. 2).

Taken together, our results indicate that the TLR3 L412F homozygosity impairs the surface expression of TLR3 and reduces the level of IFN-γ response to poly(I:C) in NK cells. Thus, TLR3 412F/F genotype could contribute to the poly(I:C)-hyporesponsiveness observed in HL subjects.

Table 2. Association of TLR3 L412F polymorphism with herpes labialis (HL). [a]P-value was calculated by one-sided Fisher' exact test for detecting difference of allele frequencies. [b]P-value was calculated by one-sided Fisher's exact test comparing frequencies of F/F homozygote with frequencies of combined heterozygote and wild type individuals in the HL and the control groups.

TLR-3 L412F	N	var. allele T (%)	P-value[a]	genotypes (%) L/L	L/F	F/F	P-value[b]
HL	51	39 (38.2)	0.08	19 (37.3)	25 (49)	7 (13.7)	0.24
control	53	30 (28.3)		25 (47.2)	24 (45.3)	4 (7.5)	

4.4.3 The poly(I:C)-induced IFN-γ response is highest in HSV-1-seronegative individuals

In previous sections we have compared the TLR responses of people with recurrent HL with TLR functions of HSV-1-seropositive asymptomatic controls. During the course of our studies we identified 6 individuals without HL histories, who were HSV-1 seronegative. Of note, 5 of the seronegative donors were TLR3 wild type, with the other one being TLR3 L412F heterozygote. Interestingly, poly(I:C)-induced IFN-γ responses in NK cells of 5 seronegative subjects tested were significantly higher than those in HSV-1-seropositive asymptomatic carriers (median 3.7% vs. 1.4%, $P < 0.01$) (Fig. 15). As described in section 4.3.2, poly(I:C) responses in people with recurrent HL were even lower than the levels observed in asymptomatic controls (median 0.5% vs. 1.4%, $P< 0.01$) (Fig. 15).

These data suggest that the TLR3 response of NK cells might be important not only in controlling symptomatic HSV-1 reactivations, but also in protecting from primary HSV-1 infections.

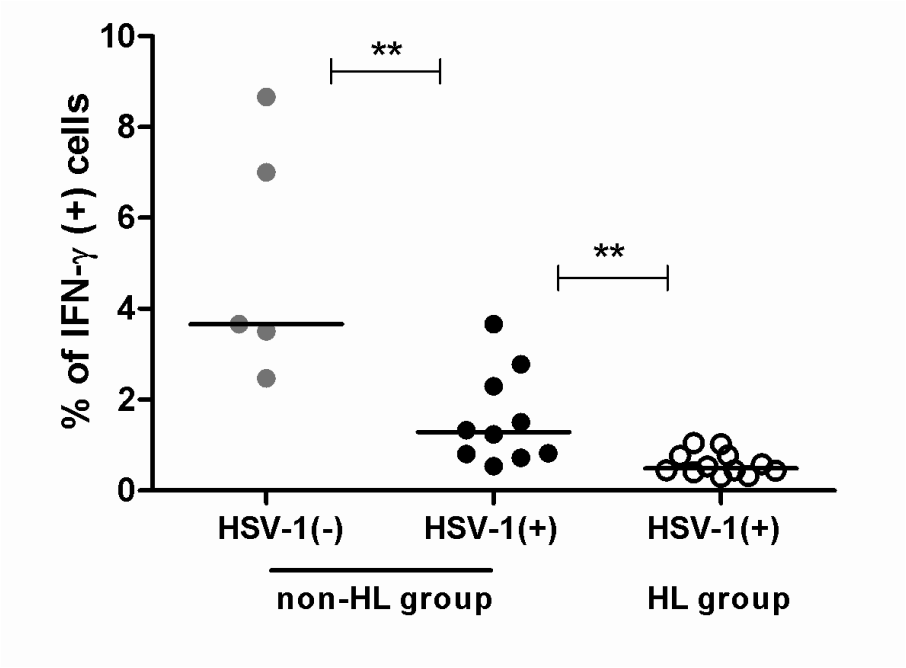


Fig. 15. Isolated NK cells derived from HSV-1-seronegaitve individuals showed highest IFN-γ responses to poly(I:C). Isolated NK cells were stimulated with poly(I:C) in the presence of 1 ng/ml IL-12 for 24 h. Gray dots: HSV-1 seronegative healthy donors; black dots: HSV-1-seropositive, asymptomatic individuals; open circles: HSV-1-seropositive subjects with recurrent HL. **P<0.01 by Mann-Whitney U tests. Lines represent medians.

4.4.4 Purified NK cells can be infected by HSV-1 when activated

It is known that HSV-1 proteins interfere with cellular interferon- and TLR- responses, thereby promoting viral replication [90,91,92]. In order to exclude that the impaired TLR3-induced IFN-γ response in people with recurrent HL was merely due to the inhibitory effect of HSV-1 infection in NK cells, we conducted an HSV-1 infection assay on purified NK cells. By using a recombinant HSV-1 which contains GFP-conjugated Us3 gene, we showed for the first time that isolated NK cells could be infected by HSV-1, after a 24-hour pre-activation with IL-2 and PHA. Flow-cytometric analysis revealed an infection rate of 15.1% and 15.3% in 2 donors analyzed (Fig. 16).

Taken together, these results suggest that NK cells are under most stimulation conditions resistant to HSV-1 infection. Therefore, the reduced TLR3 response observed in NK cells of HL subjects is less likely to be due to the suppressive effect of direct HSV-1 infection.

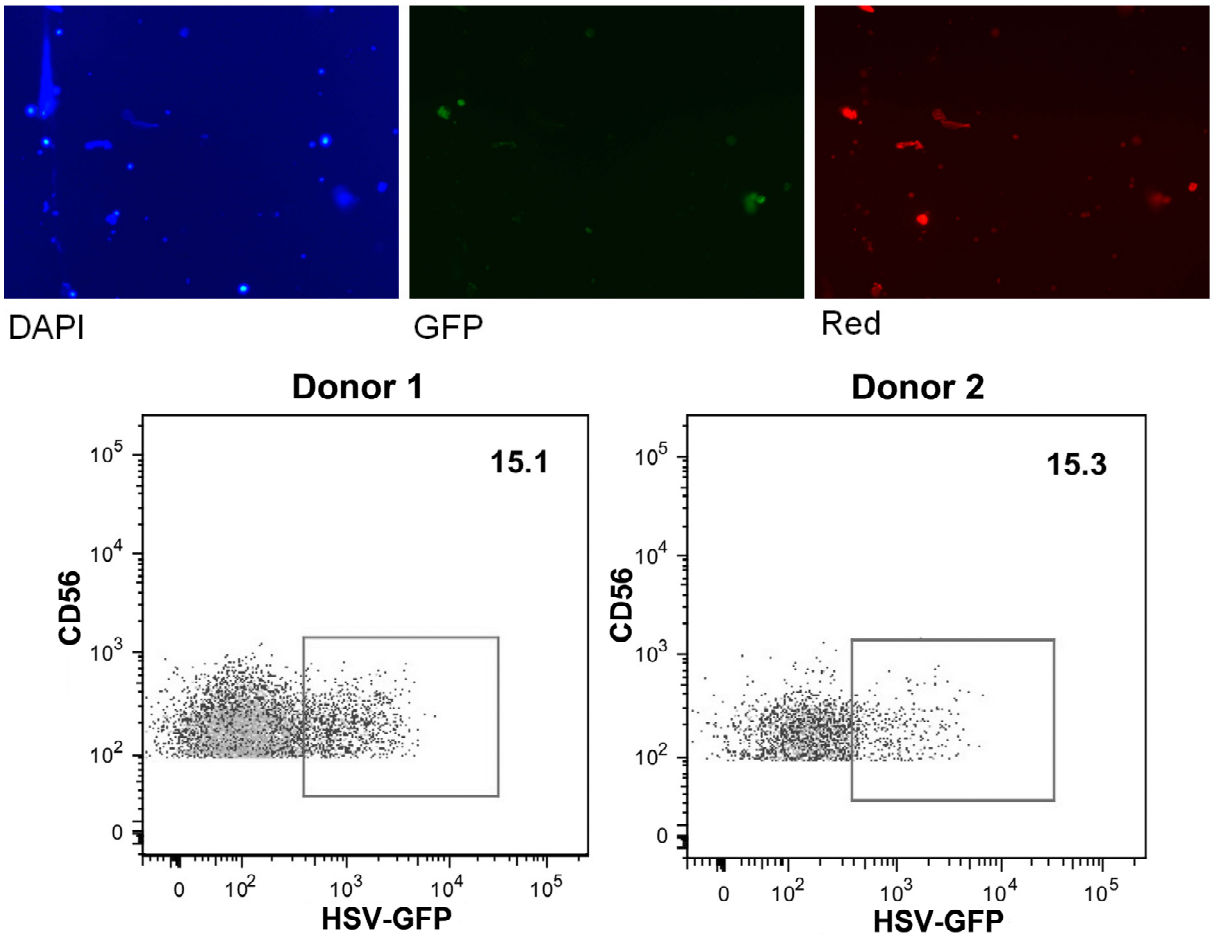


Fig. 16. HSV-1 infection of purified NK cells. . Isolated NK cells were infected with GFP-expressing recombinant HSV-1 (MOI=25) after pre-activation with IL-2 and PHA for 24 h. Infected NK cells were examined via fluorescence microscope (upper panel) and flow cytometry (lower panel). Dot plots of NK cells derived from 2 different donors are shown in the lower panel. The percentage of GFP-positive, and therefore infected, NK cells is given in the upper right corner.

4.4.5 The cytotoxic potential of NK cells in HL subjects is not impaired

Reduced NK cell percentages and diminished TLR3-induced IFN-γ production were the most prominent findings we observed in subjects with recurrent HL. To address the possibility that other NK cell effector functions in HL subjects were also impaired, we studied the cytotoxic potential of isolated NK cells in 2 HL subjects whose NK cells showed hyporesponsiveness to poly(I:C) stimulations, and compared the results with those of 2 asymptomatic healthy controls whose NK cells were highly responsive to all TLR agonists. Cytotoxic response was detected by degranulation as assessed by CD107a surface expression after co-incubations of IL-12-preactivated NK cells with the

NK-target K562 cells (Fig. 17). The IL-12-enhancecd increase of CD107a levels was not lower in the 2 HL subjects (low responders) as compared with the 2 controls (high responders) (Fig. 17A-B). However, in accordance with a previous study [57], further addition of the TLR3 agonist did not increase the IL-12-induced cytotoxicity in both groups (shown as a representative histogram in Fig. 17C).

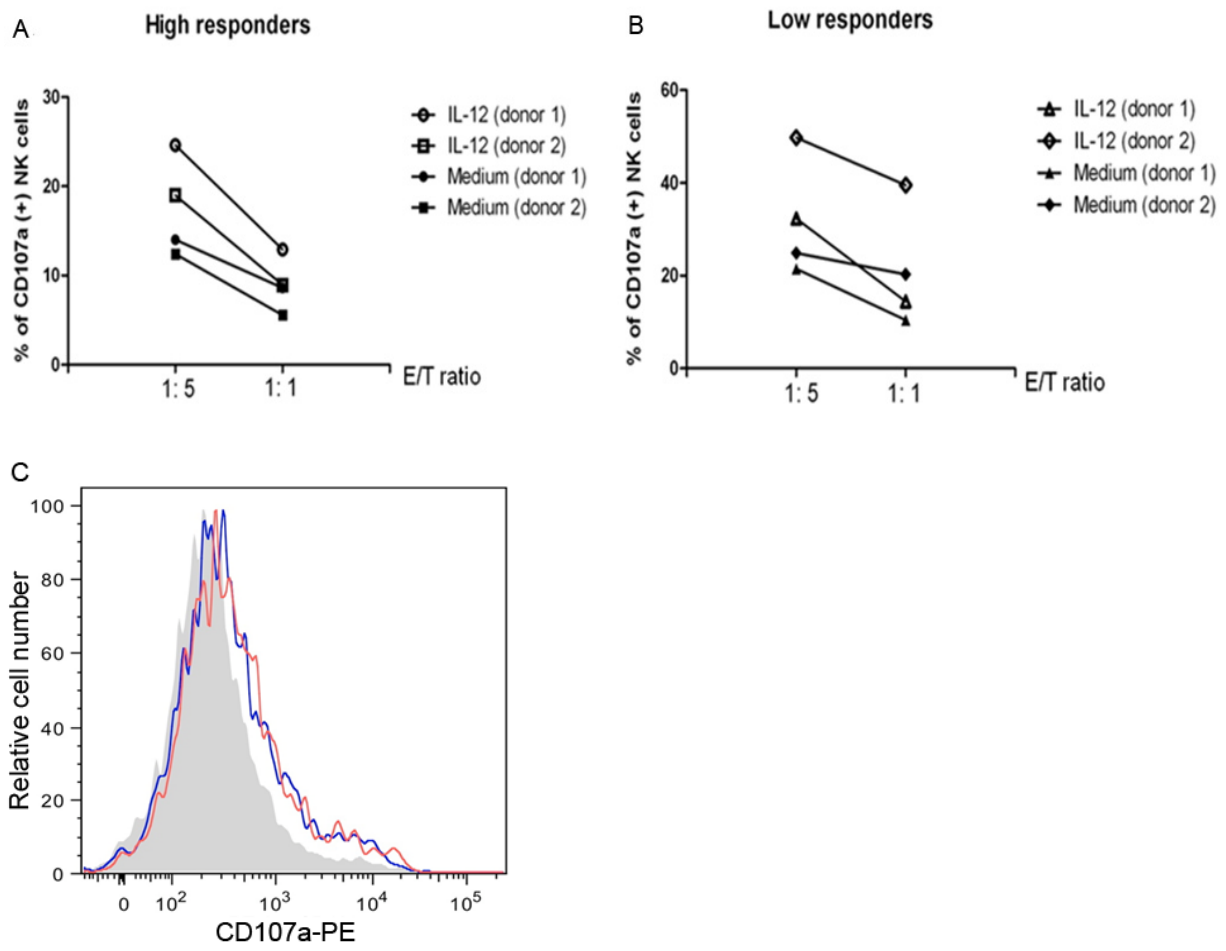


Fig. 17. Cytotoxic degranulation as determined by CD107a expression on isolated NK cells of healthy controls and HL subjects. Degranulations of isolated NK cells from **(A)** 2 TLR high responders (asymptomatic controls) and **(B)** 2 TLR low responders (individuals with HL history) are shown. E/T ratio: effector (NK cell) / target (K562 cell) ratio. **(C)** Representative flow-cytometirc histogram of NK cell CD107a expression in one donor. Shaded curve: unsimulated NK cells; red curve: stimulated with 1 ng/ml IL-12; blue curve: stimulated with IL-12+poly(I:C).

4.5 TLR-induced IFN-γ responses of CD56^{bright} NK cells in HL subjects

As mentioned in the introduction, NK cells can be divided into CD56^{dim} and CD56^{bright} subsets. In the first part of the result section we also showed that although CD56^{bright} NK cells are the minor population (1.4%-7.3% of total NK cells), a large proportion of these cells produce IFN-γ in response to TLR agonist stimulations. Since we have observed reduced TLR3-induced IFN-γ in total NK cells of individuals with recurrent HL, we wanted to further analyze the TLR-triggered IFN-γ responses in the CD56^{bright} subset.

4.5.1 Reduced IFN-γ response of CD56^{bright} NK cells to R-848 in people with HL

In resting NK cells, the percentage of CD56^{bright} subsets is similar between HL subjects and asymptomatic controls (Fig. 18A). Remarkably, in addition to the diminished response to poly(I:C), CD56^{bright} NK cells of individuals with recurrent HL showed also reduced IFN-γ response to the TLR7/8 agonist, R-848 (median of % of IFN-γ-positive cells, HL vs. control: 27.0% vs. 49.5%, P = 0.02; Fig. 18B). In contrast, the TLR responses of CD56^{dim} cells were similar to those observed in total isolated NK cells, since they represent the majority of NK cell population: CD56^{dim} cells of HL subjects only showed lowered IFN-γ response to poly(I:C), but not to R-848 (Fig. 18B).

Furthermore, in order to examine if the IL-12 profiling for R-848 stimulation already led to differential up-regulation of CD56 molecule on HL subjects and controls, we analyzed the percentage of CD56^{bright} subset after the addition of 1 ng/ml IL-12 alone. No difference was observed between asymptomatic controls and people with recurrent HL (Fig. 18C).

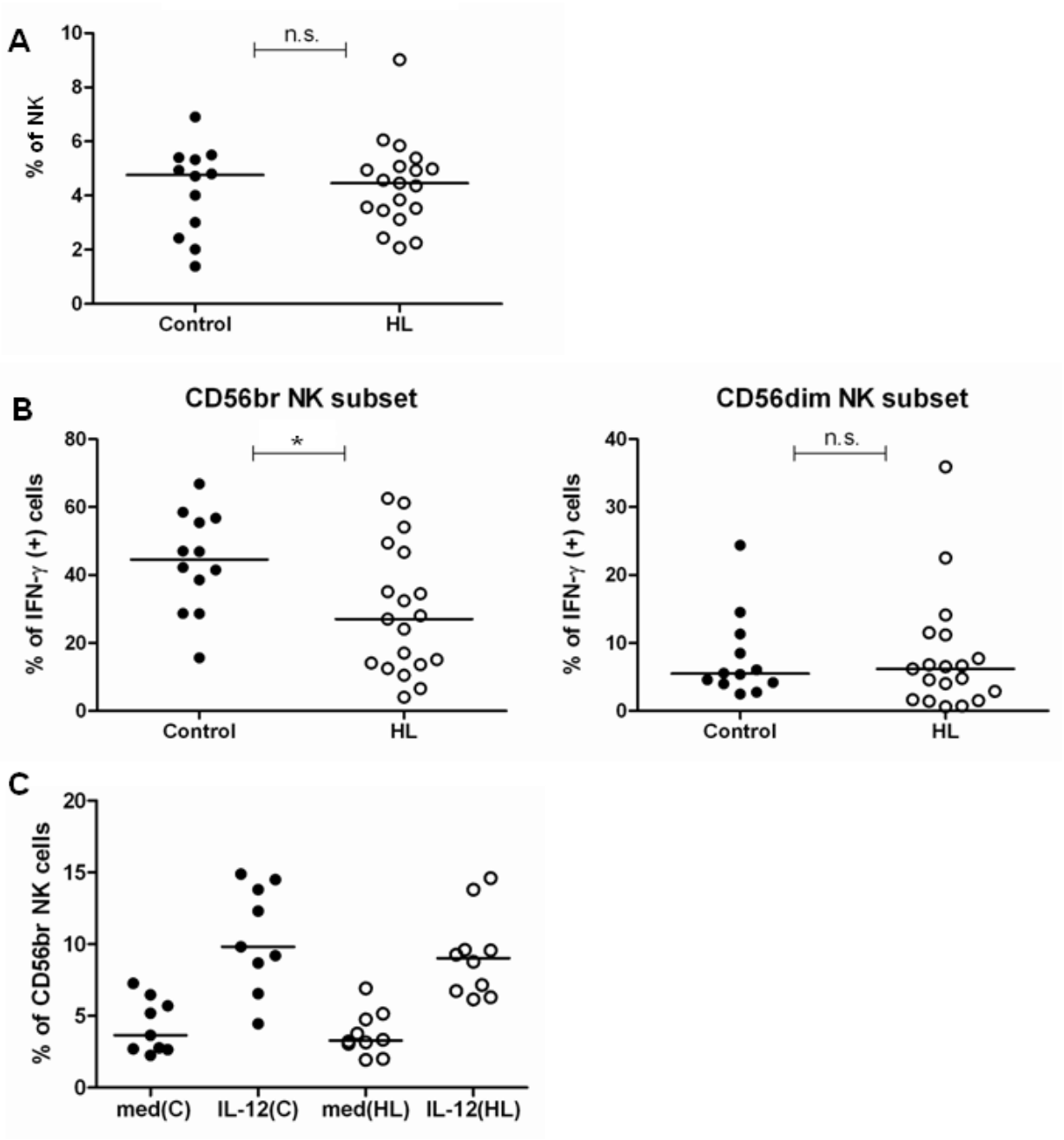


Fig. 18. Reduced IFN-γ response to R-848 in CD56^{bright} NK cells of HL subjects. (A) Percentage of CD56^{bright} subset in total NK cells. **(B)** IFN-γ response to R-848 in CD56^{bright} and CD56^{dim} subsets of asymptomatic controls (n = 12) and of people with recurrent HL (n = 19). *P< 0.05 by Mann-Whitney U test. Lines indicate medians. **(C)** Up-regulation of CD56 after 1 ng/ml IL-12 profiling in purified NK cells of controls (C) and HL subjects. Med: medium only.

4.5.2 Co-stimulatory function of accessory cells restored the reduced IFN-γ response of CD56^{bright} NK cells to R-848

Similar to the findings of poly(I:C)-hyporesponsiveness in total NK cells, the diminished R-848 response of CD56^{bright} NK cells derived from HL subjects could be restored when assessed within PBMCs (Fig. 19A). Co-incubation experiments of NK cells with monocytes, DCs, or supernatant of R-848-activated PBMCs were performed to determine the co-stimulatory factor. Interestingly, besides the stimulating effect of monocytes and DCs, the addition of R-848-activated supernatant alone could largely increase the IFN-γ response of CD56^{bright} NK cells in HL subjects (Fig. 19A).

These results suggest that soluble factors secreted from accessory cells could restore the reduced R-848-response in CD56^{bright} subsets. Since IL-15 has been reported to be important in enhancing NK cell activity against *in vitro* HSV-1 infection [93], we added 10 ng/ml of IL-15 to isolated NK cells derived from 3 R-848-low responders during R-848 stimulation. As shown in Fig. 19B, IL-15 could increase the R-848-responsiveness of CD56^{bright} NK cells in these individuals.

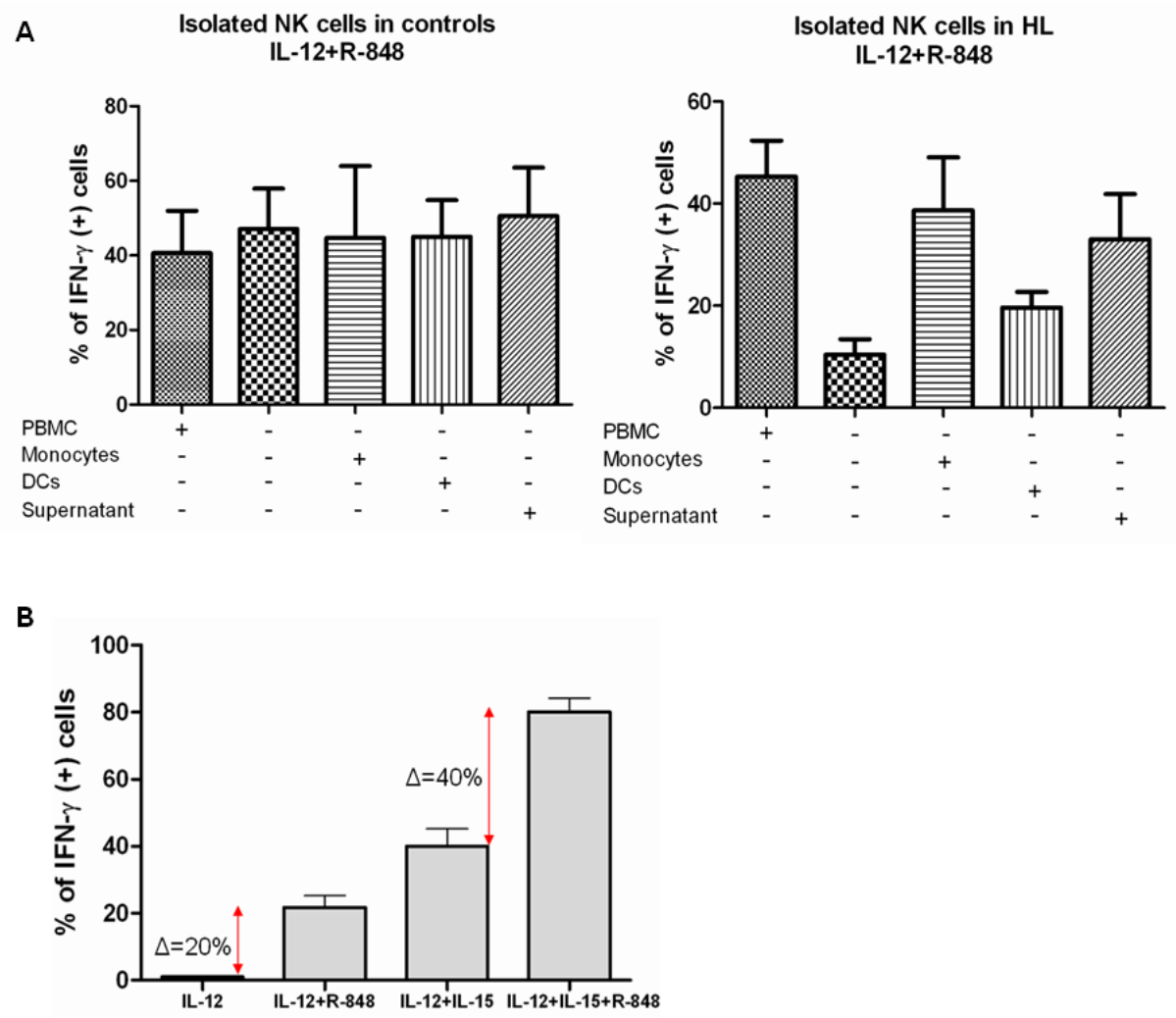


Fig. 19. Soluble factors from accessory cells could restore the R-848-hyporesponsivenss of CD56^{bright} NK cells. (A) IFN-γ responses to R-848 in CD56^{bright} subset of isolated NK cells were shown. In the HL group, addition of monocytes, DCs, and supernatant from R-848-activated PBMCs increased the level of CD56^{bright} cell response to R-848. n = 5, error bars represent mean±SEM. **(B)** The addition of IL-15 enhanced the CD56^{bright} NK cell R-848 responsiveness in R-848-low responders. N =3, error bars represent mean±SEM.

4.6 IFN-γ response of NK cells to HSV-1

In previous sections we showed that NK cells could be infected by HSV-1. Moreover, we demonstrated that TLR3-hyporesponsiveness in total/CD56^{dim} NK cells and TLR7/8-hyporesponsiveness in CD56^{bright} NK cells were associated with susceptibility to recurrent HL. Therefore, we wanted to further analyze if the IFN-γ response of NK cells to HSV-1 is impaired in HL subjects. Supernatants of HSV-1-uninfected and -infected NK cells were collected for detection of IFN-γ. However, we could not evaluate the

HSV-1 effect; because in order to infect NK cells with HSV-1, purified NK cells need to be pre-activated by IL-2 and PHA, which are already strong IFN-γ stimuli.

Hence, instead of using live HSV-1, we stimulated IL-12-primed PBMCs and IL-12-activated isolated NK cells with 10 μg/ml UV-inactivated HSV-1 viral lysate or with control cell lysate for 24 hours. NK cells ($CD56^{dim}$ and $CD56^{bright}$ subsets) analyzed within PBMCs of both the asymptomatic controls and the HL group produced substantial amount of IFN-γ in response to HSV-1 lysate (Fig. 20A). Like TLR responses of NK cells detected within the PBMC, no difference in response to HSV-1 was observed between controls and HL subjects.

However, when NK cells were isolated, no or rather low IFN-γ responses to HSV-1 were detected in both groups even in the presence of 1 ng/ml IL-12 (Fig. 20B). The result suggests that other co-stimulatory signals are needed to fully activate purified NK cells to respond to the UV-inactivated HSV-1 lysate. Thus, 1 ng/ml of IL-15, another important co-stimulatory cytokine for NK cells, was added to the HSV-1 stimulation. The combination of IL-12 and IL-15 did enhance the $CD56^{bright}$ NK cell response to HSV-1 prominently in 3 of the 6 individuals studied (Fig. 20C). Two of the 3 subjects, whose isolated NK cell showed enhanced response to HSV-1 after IL-12 and IL-15 priming, belonged to the asymptomatic control group. The other subjects all had a history of recurrent HL.

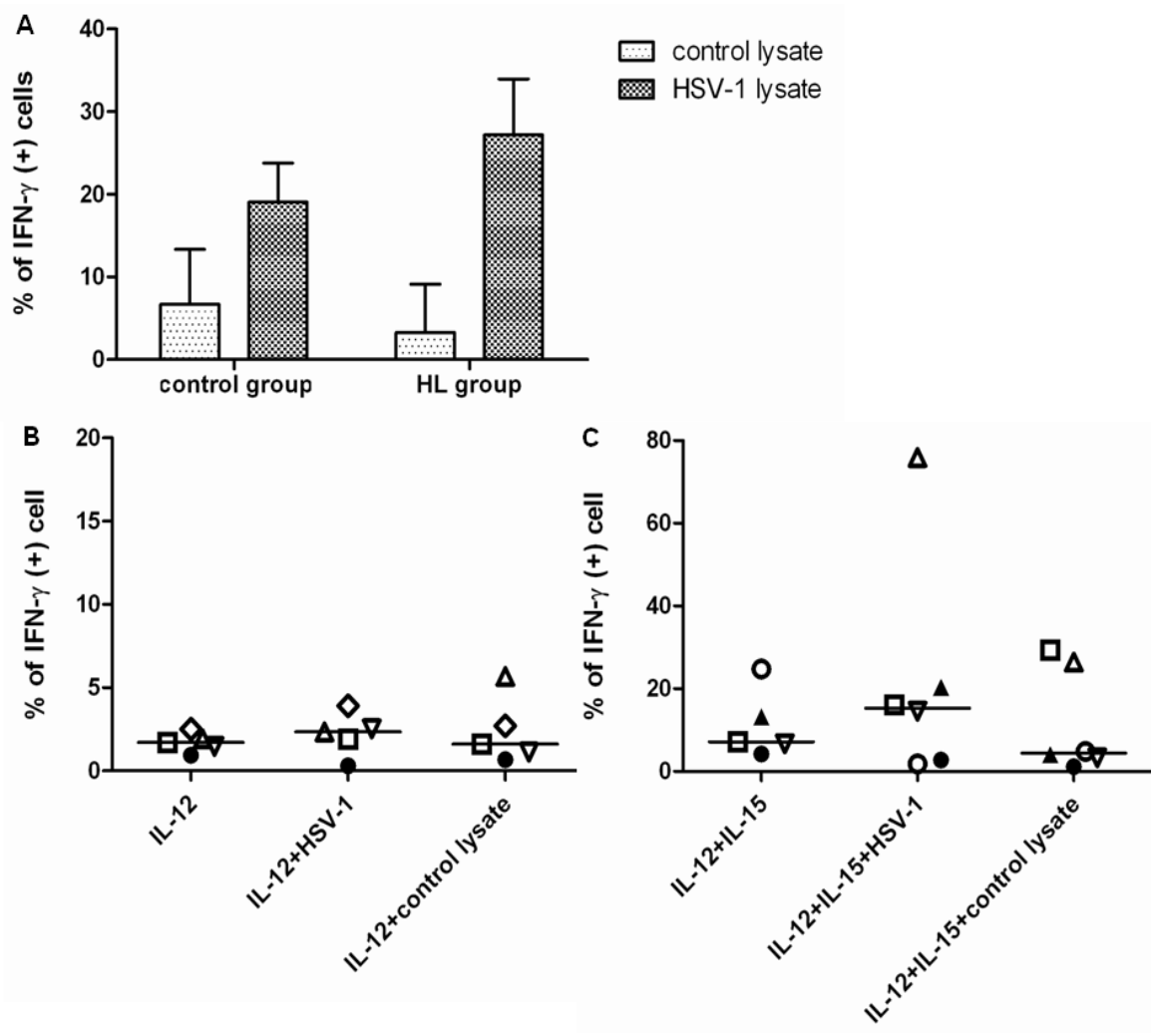


Fig. 20. HSV-1 lysate-induced IFN-γ production in NK cells. **(A)** IFN-γ production of IL-12-activated, $CD56^{bright}$ unfractionated NK cells in response to 24h-HSV-1 stimulation. The background level of IFN-γ stimulated by 1 ng/ml IL-12 was subtracted. Control group: n=4, HL: n=4. Mean± SEM is presented. **(B)** IFN-γ responses to IL-12+HSV-1 stimulation in $CD56^{bright}$ subset of isolated NK cells. Each symbol represents a different individual. Lines represent medians. **(C)** IFN-γ production of IL-12 and IL-15-primed isolated NK cells in response to HSV-1 lysate stimulation. Responses of $CD56^{bright}$ NK cells are shown. Lines represent medians.

Part III Functional impact of TLR1I602S on NK- and T- cells and association with gastric cancer

4.7 TLR1 602S/S genotype decreases IFN-γ response to Pam3Cys in NK- and CD8+T cells

In our studies, we did not detect a significant difference in TLR2/1 responses of NK cells between asymptomatic controls and subjects with recurrent HL; however, we had observed a wide range of NK cell IFN-γ responses to the TLR2/1 agonist Pam3Cys. It is known that TLR1 I602S SNP is highly prevalent in the European population, with an allele frequency of 75%; and it has been reported to impair the function of TLR2/1 complex [94,95]. In fact, it is the most common SNP affecting TLR function identified to date [40]. Monocytes derived from subjects homozygous for 602S were found to have decreased TNF-α production in response to Pam3Cys, due to impaired receptor trafficking [40]. Therefore, in order to find out if TLR1 I602S SNP is correlated with the differential Pam3Cys-induced IFN-γ responses observed in our assay, we investigated the impact of TLR1 I602S on TLR2/1-triggered IFN-γ production in healthy individuals of different genotypes, irrespective of their histories of HL.

Interestingly, IL-12-activated NK cells detected in the PBMCs of TLR1 602S/S homozygous individuals showed significantly lower frequency of IFN-γ-producing cells, compared with people of heterozygous and wild type genotypes ($p<0.001$) (Fig. 21A). Similarly, CD8+T cells of people with 602S/S genotype secreted significantly less IFN-γ in response to the TLR2/1 agonist, compared with donors of 602S/I and 602I/I genotypes combined ($p = 0.02$) (Fig. 21B). In order to rule out indirect TLR responses mediated by accessory cells within PBMCs, NK cells were further isolated. Consistently, isolated NK cells of 602S/S individuals demonstrated reduced IFN-γ production after Pam3Cys stimulation, as compared with those of the other TLR1 genotypes (median of % of IFN-γ-producing NK cells: 0.6% vs. 7.8%; $p = 0.007$) (Fig. 21C). We could not analyze the Pam3Cys response in isolated T cells since T cells require the co-stimulatory function of antigen presenting cells to respond to Pam3Cys (please refer to section 4.1.4).

In accordance with previous report [40], monocytes derived from donors with 602 I/S and 602 I/I genotypes produced significantly larger amount of TNF-α in response to 10

ng/ml Pam3Cys, as compared with monocytes of 602 S/S donors (P = 0.02, Fig. 22). The difference became less prominent at higher Pam3Cys concentrations (Fig. 22).

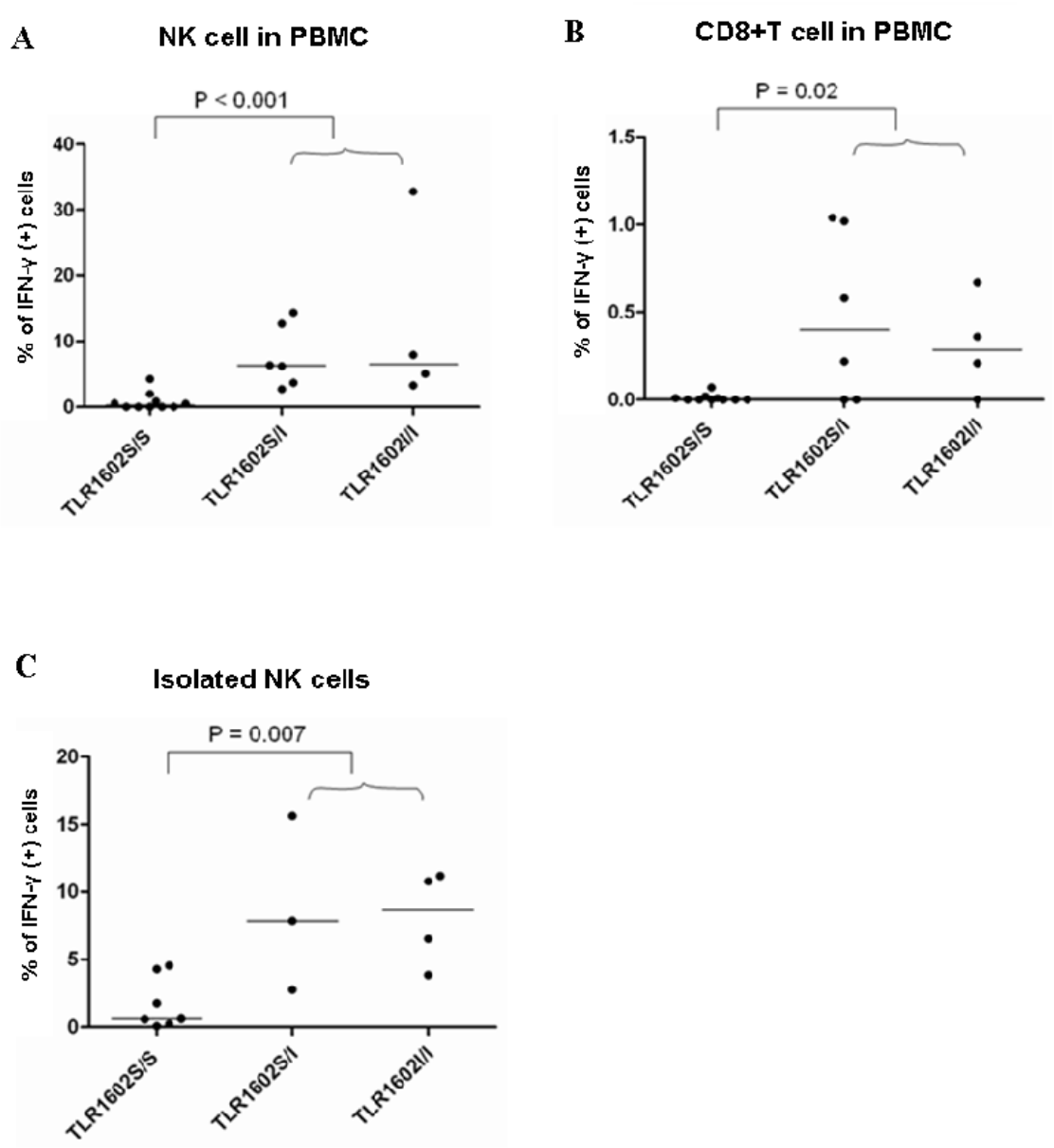


Fig. 21. TLR2/1-induced IFN-γ production is impaired in NK cells and CD8+T cells of TLR1 602S/S homozygous people. (A) and **(B):** IFN-γ responses were detected in NK cells and CD8+T cells within the whole PBMCs via flow cytometry. TLR1 602S/S, n=10; TLR1 602S/I, n=6; TLR1 602I/I, n=4. **(C)** IFN-γ responses of isolated NK cells derived from donors with different TLR1 genotypes. TLR1 602S/S, n=7; TLR1 602S/I, n=3; TLR1 602I/I, n=4. Two-tailed exact P value was determined by Mann-Witney U test comparing homozygous versus combined heterozygous and wild type donors. The lines represent median values. Background IFN-γ production in cells incubated with IL-12 alone was subtracted.

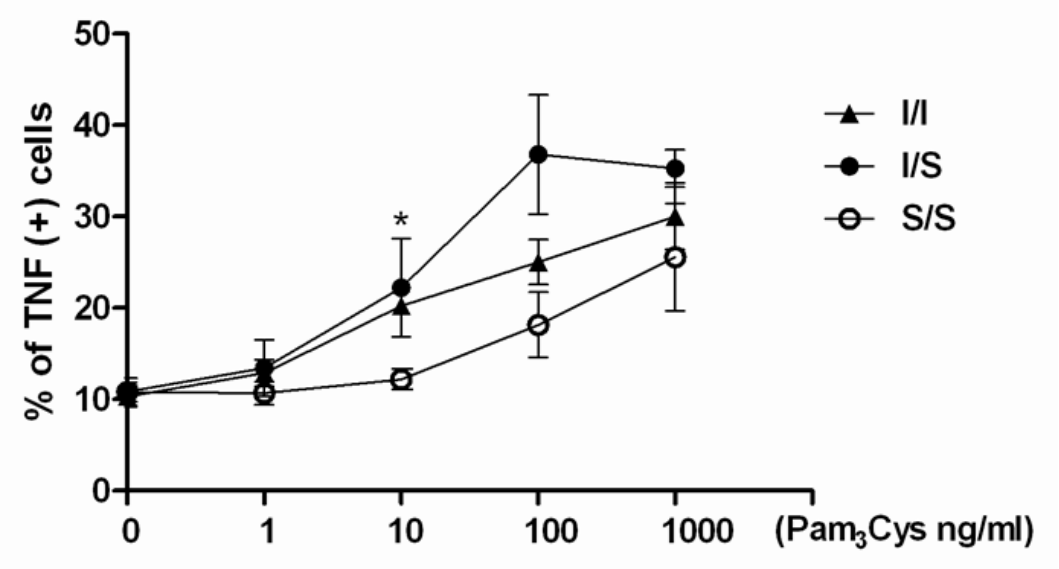


Fig. 22. TNF-α production by monocytes of different TLR1 genotypes in response to Pam3Cys. Monocytes were stimulated with other cells of PBMC for 4 hours with medium only (Med) or with different concentrations of Pam3Cys for 4 hours. Each dot represents mean ± SEM, n=3. Mann-Whitney U test was performed to compare the responses between the homozygous people and the combination of individuals with wild type and heterozygous alleles. *: $P < 0.05$

4.8 TLR1 I602S SNP and susceptibility to recurrent HL

It has been reported that DCs recognizes HSV via TLR9 and TLR2, which forms hetero-dimmers with TLR1 or TLR6 [6]. Therefore, although we did not observe TLR2/1 hyporesponsiveness in NK cells derived from HL subjects, we genotyped 20 HL subjects and 22 asymptomatic controls for TLR1 I602S SNP (Table. 3). In accordance with the functional data, the frequency of TLR1 602S allele was not higher in HL subjects.

Table 3. Association of TLR1 I602S polymorphism with HL. [a] P-value was calculated by Fisher's exact test for difference in allele frequencies. [b] P-value was calculated by chi-square test for differences in genotype frequencies.

TLR-1 I602S	N	var. allele G (%)	P-value[a]	genotypes (%) I/I	I/S	S/S	P-value[b]
HL	20	29 (65.9)	0.82	3 (15)	9 (45)	10 (50)	0.94
control	22	25 (62.5)		3 (13.6)	9 (40.9)	8 (36.4)	

4.9 Association of TLR1 I602S and gastric cancer

As described in the introduction, IFN-γ secreted by NK cells and T cells are essential anti-tumor effectors [59,60]; and *H. pylori*, an important etiological factor for gastric cancer, can be recognized via TLR2/1 complex [36]. Therefore, the TLR1 I602S SNP impairing the IFN-γ responses of NK cells and CD8+T cells might play a role in the susceptibility to gastric cancer.

4.9.1 TLR1 602S/S genotype is associated with susceptibility to gastric cancer

With the help of Dr. Lutz Hamann (Schumann Lab, Institute for Microbiology and Hygiene, Charite, who provided table 4-6) and Departments of Gastroenerology and Hematology/Oncology in Charite University Medical Center, we genotyped 326 gastric cancer patients and 410 controls (*H. pylori*-positive, without gastric cancer) for TLR1 I602S. Another functional SNP, TLR2 R753Q, was also investigated for comparison. We did not detect any significant association of the allele frequencies with gastric cancer (Table 4). However, the presence of the homozygous TLR1 602S/S genotype was significantly associated with susceptibility to gastric cancer (P = 0.03). TLR2 753Q/Q homozygosity, on the other hand, exhibited no significant association with gastric cancer in our study.

Table 4. Association of TLR1 I602S and TLR2 R753Q polymorphisms with gastric cancer. [a] P-values were calculated by Fisher's exact test for difference in allele frequencies. [b] P-values were calculated by chi-square test for differences in genotype frequencies; [c] Some DNAs gave no results for TLR-2 R753Q genotyping.

TLR-1 I602S	N	var. allele G (%)	P-value[a]	genotypes (%) I/I	I/S	S/S	P-value[b]
Gastirc cancer	326	498(76.4)	0.12	27(8.3)	100(30.1)	199(61.0)	0.03
control	410	596(72.7)		30(7.3)	164(40.0)	216 (52.7)	

TLR-2 R753Q	N	var. allele A (%)	P-value[a]	genotypes (%) R/R	R/Q	Q/Q	P-value[b]
Gastirc cancer	318[c]	21(3.3)	0.49	298 (93.7)	19 (6.0)	1 (0.3)	0.54
control	410	33(4.0)		379 (92.4)	29 (7.1)	2 (0.5)	

4.9.2 TLR1 602S/S genotype is not associated with susceptibility to *H. pylori* infection

Since TLR1 is involved in the recognition of *H. pylori*, we wanted to investigate if TLR1 602S/S genotype results in higher susceptibility to *H. pylori* infection, and hence leads to an increased risk of gastric cancer. The information about the presence of *H pylori* was unavailable in this gastric cancer cohort. However, it is well known that cardia gastric cancer is not driven by *H. pylori* infection. We therefore compared the TLR1 genotype frequency between patients with cardia- and non-cardia gastric cancers (Table. 5). There was no significant difference detected, which indirectly suggests that *H. pylori*-mediated chronic inflammation might not be the cause for the observed TLR1 602S/S genotype-associated gastric cancer risk.

Table 5. TLR-1 I602S SNP is not associated with the localization of gastric cancer. [a] P-values were calculated by Chi-square test. [b] some samples of unclear localization were excluded.

TLR-1 I602S	cardia gastric cancer	non-cardia gastric cancer			
		upper third	middle third	lower third	P-value[a]
wild type	5 (7.9%)	4 (6.6%)	6 (7.1%)	9 (8.7%)	1.0
Heterozygous	20 (31.7%)	19 (31.1%)	22 (26.2%)	35 (33.7%)	0.9
homozygous	38 (60.3%)	38 (62.3%)	56 (66.7%)	60 (57.7%)	0.9
N = 312 [b]	63	61	84	104	

4.9.3 TLR1 I602S is associated with metastasis

To further characterize the impact of TLR1 I602S on the course of gastric cancer, we evaluated the association of TLR1 SNP genotype with the percentage of patients who had distant metastasis when gastric cancer was diagnosed. Remarkably, patients carrying the variant TLR1 602S allele showed increased frequency of gastric cancer metastasis at the time of diagnosis, as compared to wild type-allele carriers (P = 0.04, Table 6). Of note, the TLR1 I602S heterozygotes, who did not show elevated risks for primary gastric cancer, also exhibited higher frequencies of metatstasis in comparison to wild type patients.

Table 6. TLR1 I602S variant allele is associated with presence of metastasis. [a] P-value was determined by Fisher's exact test comparing wild type patients versus combined heterozygous and homozygous allele carriers.

TLR-1 I602S	genotypes			
	I/I	I/S	S/S	P-value [a]
With metastasis	4	39	60	0.04
Without metastasis	20	53	125	

5 Discussions

Part I About assay development

5.1 Establishment of a multi-color flow cytometry-based assay for the evaluation of TLR functions in different cell subsets of PBMC

In this study, we have established a multi-color flow cytometric assay which allowed us to detect the TLR responses in various PBMC subsets simultaneously. Previous assay developed by Deering and Orange [78] for the evaluation of TLR function assessed TLR responses in the supernatant of PBMCs, thus not allowing the attribution to cell subpopulation. In addition, multi-color flow cytometric-based assays have been described to assess TLR responses in cell subsets. To date, 4-, 6- and 8-color flow cytometric assays for TLR responses were developed [80,96,97]. However, these assays all focused on the conventional antigen-presenting cells, namely DCs and monocytes. Functional assays to determine TLR responses of NK cells have not been described so far. In viral infections, NK and T cells play an important role, and impaired DC-dependent NK cell and T cell responses to TLR ligands have been reported in human immunodeficiency virus (HIV) and hepatitis C virus (HCV) infections [98,99]. Importantly, in TLR3-deficient patients with HSE, their isolated NK cells showed impaired IFN-γ production in response to poly(I:C), but their blood DCs responded normally[26]. Therefore, by including the TLR-induced IFN-γ responses of NK cells and T cells in the panels, our assay provides the most comprehensive aspects of TLR responses, thus increasing the sensitivity of detecting potential impairments in different immune cell subsets.

In general, the TLR-induced TNF-α or IFN-γ production in each cell subset evaluated in our assay were in agreement with previous reports, except that TNF-α production of pDCs was observed after stimulation with TLR2 and TLR4 agonists, which might be due to cross talks of pDCs with other cells in peripheral blood. In T cells, the IFN-γ response was completely abrogated when BFA was added at the beginning of TLR stimulations, indicating a strong dependence of T cell-TLR responses on accessory cell cytokines. In contrast, isolated NK cells, especially the $CD56^{bright}$ subset, still exerted significant production of IFN-γ in response to TLR stimulations when BFA was added at the

beginning of TLR stimulations.

Cryopreservation of PBMCs in our experiments was possible for evaluating TLR-ligand triggered TNF-α production in monocytes, but the freeze-and-thaw procedure altered the IFN-γ responses to TLR-ligands in NK cells. Deering and Orange [78] had observed a greater TLR-ligand-induced TNF-α response in cryopreserved cells; however, we did not detect this phenomenon in our assay. It might be that instead of using the rapidly thawed cells immediately, we had rested the thawed PBMC overnight, thus decreasing the stress-induced release of cytokines.

Furthermore, large inter-individual differences in TLR-induced TNF-α and IFN-γ responses were observed in our assay. Interestingly, some donors were high-responders to certain TLR ligands, but low-responders to other TLR agonists. The individual TLR response was highly reproducible one month later. These data suggest that genetic polymorphisms of TLR pathways might underlie the heterogeneous TLR responses.

5.2 Direct TLR responses of NK cells

In accordance with previous studies [56,57,100], we demonstrated that in the presence of IL-12, TLR2/1, 3, 4, 7 and 8 agonists were able to stimulate IFN-γ secretion in purified human NK cells. In peripheral blood, the NK population is consisted of more than 90% the $CD56^{dim}$ NK subset and less than 10% of the $CD56^{bright}$ cells. These two main NK cell subpopulations show differences in both phenotypes and functions, as reviewed in [101,102]. The $CD56^{dim}$ NK cells have higher cytotoxic potentials than the $CD56^{bright}$ subset, and they express much higher levels of the Fc gamma receptor III CD16, which mediate the antibody-dependent cellular cytotoxicity efficiently. The $CD56^{bright}$ NK cells, on the other hand, express the high affinity-receptor for IL-2, and have higher capacity to produce abundant cytokines when activated. With the preactivation of IL-12 and IL-18, the $CD56^{bright}$ NK cells secrete 20-30 times more IFN-γ than $CD56^{dim}$ NK subsets [103]. Similar to these findings, we found that $CD56^{bright}$ NK cells produced over 5-fold more IFN-γ than the $CD56^{dim}$ population after TLR ligand stimulations.

Part II Association of differential TLR responses of NK cells and susceptibility to recurrent HL

5.3 TLR3- hyporesponsiveness of NK cells in subjects with recurrent HL

NK cells play essential roles in HSV infections. Both quantitative and qualitative deficiencies of NK cells have been reported to result in severe or frequent herpes virus infections [52,53]. In this study we analyzed patients suffering from the milder, however, very common symptoms of HSV-1 reactivation with lesions confined to the orolabial region. Remarkably, we observed not only lower percentages of NK cells in people with HL, but also demonstrated here for the first time that the poly(I:C)-induced IFN-γ response of purified NK cells is significantly lower in people with recurrent HL, indicating that the TLR3 response of NK cells is important for the control of HSV-1 infections.

5.3.1 NK cell hyporesponsiveness to poly(I:C) could be restored by co-stimulatory function of APCs

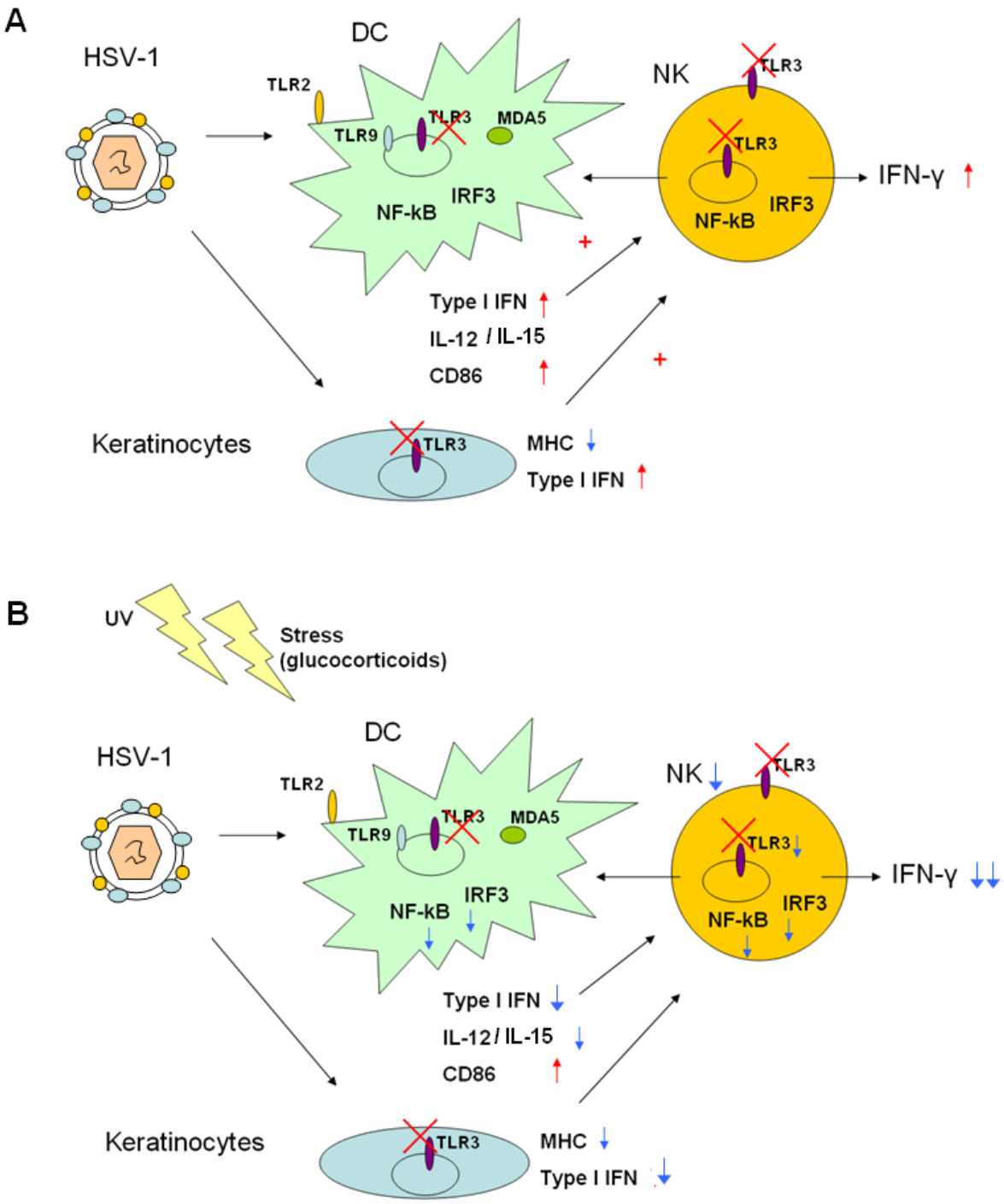
It is interesting to note that NK cells of HL subjects produced as much IFN-γ as asymptomatic controls when stimulated in the presence of other cell subsets of PBMC. We further demonstrated that the impaired poly(I:C) response of NK cells could be restored by the addition of DCs or monocytes, but not with supernatant collected from poly(I:C)-stimulated PBMCs, suggesting co-stimulatory effects of APCs via cell-contact-dependent signals or high concentrations of cytokines at the interface of APCs and NK cells.

It has been reported that poly(I:C) can trigger the cellular interferon response via TLR3 and RIG-like receptors (RLRs, including RIG-I and MDA-5) [104]. TLR3 senses dsRNA on the cell surface or in the endosome; while RLRs are cytosolic dsRNA sensors. Although we applied poly(I:C) extracellularly without transfection in our study, it is suggested that poly(I:C) could penetrate into the cytoplasm through yet unknown mechanisms [105]. APCs and NK cells express both TLR3 and RIG-I/MDA-5. However, unlike professional APCs / epithelial cells which might utilize TLR3 and other pattern recognition molecules (such as RLRs) cooperatively in response to poly(I:C) or RNA virus stimulations [104,106,107], human NK cells have been reported to recognize poly(I:C) via TLR3 only [87]. In line with these data, Zhang et al.[26] reported that

TLR3-deficient human DCs and keratinocytes could still produce substantial amount of IFN-α and -λ in response to poly(I:C), while their isolated NK cells showed impaired poly(I:C)-induced IFN-γ production. Furthermore, professional APCs such as DCs express diverse and high levels of pattern recognition molecules and thus might be more sensitive to poly(I:C) stimulations.

Therefore, we propose that although HL subjects have a deficiency in TLR3 responses; their APCs are more sensitive to poly(I:C) stimulation than lymphoid cells, perhaps by enhanced synergy between pattern recognition molecules, thus allowing them to compensate for the impaired TLR3 responses in NK cells (depicted in Scheme 2A).

It is well-known that psychological stress, UV-exposure, and superimposed infections can lead to symptomatic HSV-1 reactivation. In fact, it has been suggested that UV-exposure can down-regulate the co-stimulatory function of DCs [108]. Moreover, the stress cytokine glucocorticoid has been shown to directly suppress the TLR3 expression and poly(I:C)-induced NF-κB activation in epithelial cells [109]. NK cell numbers in stressed mice have also been found to be decreased significantly [110]. Collectively, these data suggest that people who already have a reduced NK cell number and impaired TLR3 responses might be more susceptible to suffer from HL outbreaks when under stress (depicted in Scheme 2B). In my study, I have attempted to evaluate the differential impact of UV irradiation on unfractionated NK cells of HL subjects and of controls *in vitro*, but the experiments were not successful due to technical limitations.



Scheme 2. Model of co-stimulation of APCs on restoring the impaired TLR3-induced IFN-γ in NK cells and the effects of stress. (A) HSV-1 promotes secretion of co-stimulatory cytokines in DCs and keratinocytes via TLR3-dependent and –independent pathways, which boosts the IFN-γ production in TLR3-deficient NK cells. **(B)** Stress can down-regulate cytokine secretion of co-stimulatory cells. Also, stress can directly alter TLR3 pathway and NK cell numbers, leading to further decrease of NK cell IFN-γ response to HSV-1.

5.3.2 Mechanisms of NK cell hyporesponsiveness to poly(I:C) in people with recurrent HL

Unlike the findings of Sivori et al. [88], we did not observe an association between the total amount of TLR3 mRNA/protein and NK cell response to poly(I:C). In order to find out if TLR3 SNPs might impair the level of poly(I:C)-induced IFN-γ production, we studied the functional role of TLR3 L412F. This SNP is prevalent in 25-30% of our population and has been shown to impair the NF-κB activity and interferon responses in reporter assays following poly(I:C) stimulation [89]. It has been reported to be associated with less efficient immunity after vaccination against measles virus [111] and a very recent report suggested that TLR3 L412F also plays a role in host susceptibility to coxsackievirus B3-mediated myocardidits [112]. Functional assays on NK cells of different TLR3 L412F genotypes revealed that the homozygous variant allele-carriers showed decreased IFN-γ production in response to poly(I:C), as compared to wild type. In contrast to DCs, a subset of NK cells do express TLR3 on the cell surface [87]. Very interestingly, we observed lower TLR3 surface expression in NK cells of HL subjects as well. These data are in accordance with the findings of Ranjith-Kumar et al. [89], that TLR3 L412F is under-represented on the surface of transfected 293T cells and impairs the poly(I:C) response, while the intracellular TLR3 variant level is normal. Remarkably, individuals with recurrent HL showed a higher TLR3 L412F frequency and a higher proportion of homozygous 412F/F genotype, although our analyses did not reach statistical significance. The statistics might be affected by the fact that several F/F homozygous healthy controls detected in our study are relatively young (20 – 25 years old); therefore they might develop HL later in life. Moreover, other mechanisms of susceptibility to recurrent HL exist, as discussed later in section 5.3.5.

5.3.3 Possible influences of TLR3-hyporesponsiveness on the interplay of NK cells and T cells

TLR3-hyporesponsiveness of NK cells might contribute indirectly to reduced T cell responses to HSV-1. It has been discovered that cytokines secreted by NK cells are required for optimizing the quality of HSV-1-specific CD8+T cell responses [49]. It is also shown that TLR3 is indispensible for gB-specific CD8+T cell response to HSV-1 [113], and pre-treatment with the TLR3 agonist poly(I:C) could protect human neuronal cells from HSV-1 infection [20]. Since IFN-γ secreted by CD8+T cells surrounding latently-

infected neurons was described to be the key player in preventing HSV reactivations [114,115], we propose that TLR3-deficient NK cells, which produce less IFN-γ, would reduce the number of multi-functional HSV-1-specific CD8+T cells, thus lowering the threshold of HL outbreak.

5.3.4 Highest NK cell TLR3 response in HSV-1-seronegative people

In the course of this study, we identified 6 HSV-1 seronegative donors. Of note, 5 of these individuals are TLR3 wild type, and demonstrated significantly higher NK cell IFN-γ responses to poly(I:C), as compared to the responses of HSV-1-positive asymptomatic carriers. Although we could not be sure if these individuals have encountered the virus before, chances of HSV-1 exposure due to family members with recurrent HL or lab exposure to HSV-1 were possible in 4 of the 6 subjects. It has been reported that some HSV-seronegative people could mount persistent HSV-specific T cell responses in the absence of seroconversion, suggesting viral elimination or containment of infection [116]. Whether the high TLR3-response of NK cells contributes to the resistance of HSV-1 infection requires further investigations.

5.3.5 Other possible genetic susceptibilities to recurrent HL

Our data suggest the TLR3 412F/F genotype as a risk factor of HL only for a subgroup of patients. Deficiencies of other adaptor molecules on the TLR3-signaling pathway could also contribute to the observed poly(I:C)-hyporesponsiveness. Interestingly, in an updated report written by Casanova's group [117], only 1 in 45 HSE patients sequenced for UNC93B and TLR3 genes showed the disease-predisposing mutations as previously described by the same group. However, some of these patients still displayed functional defects in the TLR3-IFN-α/-β/-λ pathway, indicating involvement of other TLR3-signaling associated genes.

Furthermore, the overlap in TLR3 responsiveness of healthy and HL people observed in our study suggests that different mechanisms underlying the susceptibility to HL exist, such as the recently described HL susceptibility locus on human chromosome 21 [24] or polymorphisms of apolipoprotein E-(APOE)-ε4 [25]. In fact, when we examined the TLR responses in the $CD56^{bright}$ NK subset, reduced IFN-γ response to the TLR7/8 agonist R-848 was observed in HL subjects. Our data suggest that functional polymorphisms in

TLR7 or 8 [118,119] might also play a role in susceptibility to recurrent HL.

Unlike the poly(I:C)-hyporesponsiveness of total NK cells, the reduced $CD56^{bright}$ cell response to R-848 could be restored by soluble factors in the TLR7/8-stimulated PBMCs. We have demonstrated that one of these co-stimulatory factors could be IL-15, a cytokine important for NK cell activation, proliferation, and control of HSV-1 infection [93,120,121]. Since monocyte is one of the main sources of IL-15 [122], it is possible that the very few contaminating monocytes (<1%) in our purified NK cell culture had diminished IL-15 response to R-848. It is known that IL-15 utilizes components of the IL-2 receptor to activate human NK cells [123]. Because the $CD56^{bright}$ NK subset has the high affinity IL-2 receptor [102], they are probably more susceptible to the minor variations of IL-15 level in culture medium. Hence, these data might explain why the R-848-hyporesponsiveness in NK cells of HL subjects was only detected in the $CD56^{bright}$ subpopulation.

5.4 NK cells and HSV-1

Our results show that TLR3 response of NK cells is associated with susceptibility to HL. However, how could NK cells recognize HSV-1 via TLR3? HSV-1 is a double-stranded (ds) DNA virus, which could produce dsRNA during erroneous converging bidirectional DNA transcription. Although TLR3 could recognize dsRNA, it has been reported that dsRNA is accumulated in the cytoplasm after HSV infection via unknown mechanisms [124]. Therefore, HSV dsRNA produced during viral replication could be recognized by cytosolic RLRs. However, unlike TLR3, which has been reported to be involved in HSV-1 encephalitis, clinical importance of RLR in HSV infections is less understood. On the other hand, it is conceivable that NK cells might sense dsRNA derived from adjacent HSV-1-lysed cells directly via cell surface TLR3, or via endosomal TLR3 through endocytosis of cell debris containing viral replication intermediates. In addition, cytosolic viral nucleic acids could be delivered to endosome via autophagy, and stimulate host interferon response [125]. Fusion of autophagosome with endosome makes the recognition of dsRNA via TLR3 possible,

We further addressed the question whether NK cells can be infected by HSV-1 at all. We show here for the first time that similar to T cells [126,127], about 15% of NK cells could be infected after pre-activations of IL-2 and PHA. It has been reported that

receptors for HSV viral entry are expressed on activated lymphocytes [128]. The requirement of PHA activation, however, suggests that NK cells, which are important in controlling HSV-1 infections, are relatively resistant to HSV-1 invasion. Since it is described that viral proteins produced during active HSV-1 infection can inhibit or lower cellular interferon responses [91], our findings that NK cells are less vulnerable to HSV-1 infection exclude the possibility that the reduced TLR3 responses observed in HL subjects were merely due to direct blocking mechanisms of HSV-1.

Moreover, we have evaluated NK cell IFN-γ responses of HL subjects and controls toward HSV-1. However, we did not succeed in analyzing live-HSV-1-induced IFN-γ, because NK cells need to be pre-activated by IL-2 and PHA, which already up-regulate IFN-γ. By using UV-inactivated HSV-1 lysate, we detected similar IFN-γ responses in PBMCs derived from asymptomatic controls and HL subjects. However, unlike the responses to TLR-ligands, isolated NK cells responded poorly to HSV-1 lysate plus IL-12, indicating the requirement of other co-stimulatory cytokines or viral replication (to produce dsRNA). The addition of IL-15 promoted IFN-γ production in response to HSV-1 lysate in 3 individuals tested. Although these 3 donors were high responders to Pam3Cys, further investigation on 22 HL subjects revealed that the functional TLR1 I602S SNP might not be associated with susceptibility to HL. Interestingly, as we later found out, these 3 individuals were of TLR1 602I/I (wild type) genotype, who exhibited higher IFN-γ response to TLR2/1 stimulations. These results suggest that although UV-inactivated HSV-1 lysate was not replicative, thus might not be able to stimulate TLR3, 7 and 8 in isolated NK cells; glycoproteins/lipoproteins on the viral surface might still serve as TLR2/1 agonist for NK cells.

Part III Functional impact of TLR1 I602S on NK cells and CD8T cells and association with gastric cancer

5.5 TLR1 I602S SNP impairs IFN-γ secretion in NK cells and T cells

TLR1 acts as a coreceptor for TLR2, and the TLR2/1 complex can recognize lipopeptide components of microorganisms. The TLR1 I602S SNP, which is prevalent in Europeans, has been shown to impair TLR1 trafficking to the cell surface, and reduce the NF-kB/TNF-α responses to Pam3Cys in transfected HEK293 cells and primary human monocytes [40,95]. In our study, we have observed Pam3Cys-hyporesponsiveness not only in monocytes, but also in NK and CD8+T cells of TLR1 602S homozygous individuals. The impaired IFN-γ response observed in both unfractionated/isolated NK cells and unfractionated T cells suggest that the TLR1 602S/S genotype could alter the function of innate and adaptive immunities. Thus, the global effect of homozygous TLR1 602S allele on immune cells led us to the suspicion that the SNP might be involved in anti-tumor defences as well. Indeed, we later found an association of TLR1 I602S SNP with susceptibility to gastric cancer.

Of note, in contrast to previous findings [40] we did not observe a down-regulation of surface TLR1 expression in TLR1 602S/S monocytes or NK cells. Previous report used flow cytometry- staining and confocal microscopy to detect the reduced surface expression of TLR1 variant. The discrepancy of my observation and their results might be partly due to the usage of different monoclonal antibodies and isotype controls for flow-cytometric detection of TLR1. Careful microscopic examinations on TLR1 expressions of NK cells, T cells, and monocytes are needed to draw further conclusions.

5.6 Association of TLR1 I602S and risk of gastric cancer

Since lipopeptide of *H. pylori*, an essential etiology for gastric cancer, can be recognized by the TLR2/1 complex [36], we investigated the frequencies of functional SNPs of TLR1 and TLR2 in patients with gastric adenocarcinoma. TLR1 I602S was found to be associated with susceptibility to gastric cancer, while TLR2 R753Q was not involved. Remarkably, we observed that the TLR1 602S/S homozygous allele carriers had significantly higher risk of gastric cancer than people with 602I/I and 602I/S genotypes.

As described in the previous section, TLR1 602S/S impairs the TLR2/1-stimulated IFN-γ responses in NK cells and CD8+T cells, thus might lead to inadequate *H. pylori* elimination. In addition, IFN-γ together with cytotoxic activities of NK-and CD8+T cells are important for tumor surveillance [129]. Expression of IFN-inducible genes in PBMCs has been compared between healthy controls and patients suffering from different cancers, including gastric adenocarcinoma, in which cancer patients showed a significantly reduced response upon stimulation with IFN-γ [130]. The authors postulated that impaired IFN-γ signaling contributes to an early, persistent and global mechanism of immune dysfunction in cancer patients. Our finding that TLR1 602S homozygous individuals who showed lowered TLR2/1-induced IFN-γ response had increased risk for gastric cancer is in line with their results.

Furthermore, we wanted to study the association of TLR1 602S SNP with *H. pylori*-related gastric cancer pathogenesis. However, the *H. pylori* status of gastric cancer patients in this retrospective study was not retrievable. Yet, it is well-established that *H. pylori* is a strong risk factor for non-cardia gastric cancer, but not for cardia gastric cancer. Hence, we examined the frequencies of TLR1 602S/S genotype in patients with cardia- and non-cardia gastric cancers. The proportion of TLR1 602S homozygous individuals was found to be similar between patients with cardia- and non-cardia gastric adenocarcinomas, which indirectly excluded the association of the TLR-1 SNP with *H. pylori* infection. Therefore, the TLR1 I602S genetic polymorphism is less likely to play a role in the pathogenicity of *H. pylori*, but might be important in affecting the efficacy of the anti-tumor immune response.

5.7 Association of TLR1 I602S and gastric cancer metastasis

In addition to the increased susceptibility to gastric cancer, we found that the TLR-1 I602S SNP is also involved in metastasis development. The variant allele carriers were at increased risk to have distant metastasis at the time of diagnosis. Unexpectedly, this correlation was observed not only in the homozygous- but also in the heterozygous-TLR1 I602S carriers, who did not exhibit an increased susceptibility for primary gastric cancer.

It is possible that although the TLR1 602I/S individuals showed comparable IFN-γ and TNF-α production to wild type after *in vitro* Pam3Cys stimulation, they might have

reduced immune responses within the *in vivo* tumor-microenvironment. Once cancer is established and has been rapidly proliferated, endogenous molecules released from damaged tissue or necrotic tumor cells could also act as ligands for TLR2 and TLR4 [131]. It has been reported that high-mobility-group box 1 (HMGB1), an alarmin protein released from dying tumor cells, could mount effective anti-Glioblastoma multiforme (an aggressive primary brain tumor) immune response via TLR2 activation [37]. These anti-tumor responses included infiltration of myeloid DCs and clonal expansion of anti-tumor T cells. Therefore, the TLR1 I602S heterozygous subjects might already show some degree of impaired TLR2/1 responses in yet unknown aspects in response to endogenous gastric cancer-associated TLR2/1 ligands.

5.8 Conclusion

TLRs are differentially expressed on PBMC subsets. With our multi-color flow cytometric assay, we were able to detect potential impairment of TLR function in each cell type simultaneously. Due to its indispensible roles in herpes virus infections and cancer immunology, we focused specifically on TLR responses of NK cells. A large variation of TLR responses was observed in different individuals. The heterogeneous TLR-triggered IFN-γ responses in NK cells could result in differential susceptibility to HSV-1 diseases or cancer.

In the second part of our study, we have observed both quantitative and qualitative defects in NK cells of people with recurrent HL. The poly(I:C)-induced IFN-γ responses were highest in NK cells derived from HSV-1-seronegative donors, followed by responses of asymptomatic HSV-1 carriers, and were lowest in people with symptomatic orolabial reactivations. The strong correlation of TLR3 responses of NK cells with the status of HSV-1 infection suggests that polymorphisms of molecules in the TLR3 signaling pathway (i.e. as described in [89,111]) might be essential factors for the increased susceptibility to HL. We have demonstrated that TLR3 L412F SNP could be one of these factors, which impairs the surface TLR3 expression and poly(I:C)-mediated IFN-γ responses of NK cells. Reduced IFN-γ production to R-848 was observed in the $CD56^{bright}$ NK cell sub-population of HL subjects, which could be restored by soluble factors secreted by monocytes or DCs, such as IL-15. Therefore, polymorphisms in TLR7/8 or IL-15 might play a role in susceptibility to HSV-1 reactivations as well.

In the third part of our study, we found that the TLR1 602S homozygous allele carriers showed not only decreased TNF-α responses to Pam3Cys in monocytes, but also significantly reduced IFN-γ responses in their NK cells and CD8+T cells. In a limited number of patients with recurrent HL, we did not observe a higher frequency of TLR1 I602S SNP. However, associations of TLR1 I602S polymorphism with risks for gastric cancer and metastasis were detected.

Our results suggest that genetic polymorphisms of TLRs can impair TLR function of NK cells, which contribute to the increased susceptibility to HL and gastric cancer. Further research on the means of boosting TLR3 responses of NK cells might provide a novel clinical therapy for patients with symptomatic HSV-1 reactivations. Moreover, studies on enhancing TLR1 responses might aid in developing strategies against gastric cancer progression.

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Selbständigkeitserklärung

Hiermit erkläre ich, Chin-An Yang, dass ich die vorliegende Doktorarbeit mit dem Thema: „Characterization of differential Toll-like receptor function in human immune cells and association with susceptibility to recurrent HSV-1 reactivations and gastric cancer“ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Datum Unterschrift